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(54) Title: VACCINATION OF HIV INFECTED PERSONS FOLLOWING HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

(57) Abstract: The present invention provides a method of permitting cessation of antiviral therapy on HIV-infected subjects without virus rebound or with at least a delayed virus rebound or a decreased post rebound set-point. The method comprises the re-induction of HIV-specific immune responses using a vaccination strategy to induce both humoral and cell-mediated immunity. The present invention achieves an immunological control of persistent infectious virus after discontinuation of antiviral therapy. The vaccine strategy according to the invention is both safe and immunogenic in the subject HIV-infected patient population.

VACCINATION OF HIV INFECTED PERSONS FOLLOWING HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the field of methods of treating HIV-infected patients.

Summary of the Related Art

HIV infection is characterized by high levels of virus replication at all stages of infection. Virus replication causes increased levels of CD4 cell destruction and turnover, and when unchecked, immunodeficiency, AIDS and death. This model of pathogenesis has prompted a dramatic change in the treatment paradigm which has evolved from late intervention in symptomatic individuals to a "hit early, hit hard" strategy.

Perelson and co-workers developed a mathematical model based on the biphasic decay of plasma HIV RNA after initiating potent antiviral therapy. The model hypothesized that two to three years of treatment with a completely suppressive regimen could result in a virologic remission or "eradication of infection" in HIV-infected individuals. The two to three year estimate required complete suppression of virus replication, the absence of any additional slower decaying compartments and/or the absence of sequestered areas of virus replication.

Subsequently, it has been demonstrated that a pool of latently infected resting CD4+ T-cells harboring infectious provirus persists in individuals treated with highly active antiretroviral therapy (HAART). The decay characteristics of this compartment remain somewhat controversial. Finzi and coworkers have performed longitudinal quantitative HIV-1 co-culture studies on HAART treated subjects. They have concluded that this pool decays with an average half-life of 44 months. Studies by Zhang et al and Ramratnam et al suggest that the inherent decay rate of the latent pool is much shorter and is approximately 6 months on average. Given these decay rates, eradication with antiviral therapy alone would require a minimum of 10 years of complete suppression of viral replication.

Ramratnam and co-workers demonstrated that in individuals exhibiting prolonged decay characteristics of the latent pool, ongoing virus replication was evident. Other investigators have come to similar conclusions by measuring markers of ongoing replication including HIV-1 mRNA species in PBMC and levels of 2LTR circles in PBMC. As would be predicted, attempts to discontinue therapy in apparently well-suppressed individuals have been associated with virologic rebound within days to weeks of therapy discontinuation. Furthermore, it has been observed that the initial rate at which the plasma viremia increases (doubling time) is somewhat uniform and generally observed to be approximately 1.5 days.

The use of combination antiretroviral therapy has markedly altered the natural history of HIV-1 infection. Both HIV-1-related mortality and morbidity have been significantly reduced by the introduction of combination antiretroviral therapies including potent inhibitors of HIV protease and reverse transcriptase [Palella, 1998]. Despite these gains, however, it is clear that these therapies are less than ideal. Long term antiretroviral therapy is associated with significant toxicities, both short term and long term [Carr, 1998; Carr, 1998; Sulkowski, 2000; Vigouroux, 1999; Brinkman, 1999; Echevarria, 1999]. Perhaps most disturbing are the metabolic consequences of long term therapy. Syndromes including hyperlipidemias with the potential for accelerated atherosclerosis, disfiguring peripheral fat and muscle wasting and central fat deposition, as well as hyperglycemia and glucose intolerance has been associated with long term antiviral therapy. Furthermore, it is clear that the current therapies require a degree of patient adherence that is often difficult to achieve. The result of non-adherence is treatment failure and may allow for the emergence of drug resistant viruses. Therefore, treatment strategies designed to limit the duration of antiviral therapy are clearly desirable.

SUMMARY OF THE INVENTION

The present invention provides a method of permitting cessation of antiviral therapy on such HIV-infected subjects without virus rebound, with a delayed viral rebound, or with decreased post-rebound set point. The method comprises the re-induction of HIV-specific immune responses using a vaccination strategy to induce both humoral and cell-mediated immunity. The present invention achieves an immunological control of persistent infectious virus after discontinuation of antiviral therapy. The vaccine strategy according to the invention is safe and induces immune responses in the HIV-infected patient population.

The present invention is directed to a method of stimulating efficient CD4+ and CD8+ responses in a human infected with an HIV retrovirus who has a viral load of less than 10,000, preferably less than 5,000, viral copies per ml of plasma and a CD4+ T-cell count of above 300 cells/ml, preferably above 500 cells/ml, and who has been treated with a potent combination of antiviral agents that contributed to a lower viral copy number and equal or higher CD4+ cell count than before treatment. The method comprises administering a nucleic acid-based vaccine that enters the cells and intracellularly produces HIV-specific immunogens for presentation on the cell's MHC class I and MHC class II molecules in an amount sufficient to stimulate HIV-specific CD4+ and CD8+ T-cell responses, thereby reversing the otherwise observed population decline of these cells during antiretroviral therapy. In a preferred embodiment, the human has been treated with HAART therapy that resulted in the human having a viral load of less than

1,000 viral copies per ml of blood serum and a CD4+ cell count of above 500 cells/ml.

The method employs a vaccine that is a nucleic acid-based vaccine comprising naked or vectored nucleic acid. According to a preferred embodiment, the vaccine comprises an attenuated recombinant poxvirus, particularly NYVAC or ALVAC, that includes one or more nucleic acids encoding more or more HIV-specific immunogens. The vaccine optionally further comprise an adjuvant and is administered one or multiple times. The vaccine is optionally combined with an HIV antigen as well as immunostimulatory or co-stimulatory molecules such as interleukin 2 or CD40 ligand, respectively, in an amount that is sufficient to potentiate T-cell responses, in particular CD8+ responses.

The method of the invention is particularly useful for people who have been infected by HIV and who have demonstrated CD4+ and/or CD8+ T cell responses to HIV antigens, such as people who have demonstrated proliferative T-cell responses to gp120 envelope protein or p24 or both gp120 envelope and p24 Gag antigen. But the method of the invention is also useful for people who have lost their CD4+ and/or CD8+ T cell responses to HIV antigens, such as people who have lost their proliferative T cell response to gp120 or p24.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 displays plasma RNA and CD4+ T-cell levels for HIV-infected patients undergoing HAART.

Figure 2 is a bar graph displaying the number of HIV-infected subjects undergoing HAART having plasma HIV RNA levels of less than 200, 50, and 25 copies/ml.

Figures 3A and 3B display CTLp frequencies for two patients undergoing HAART.

Figures 4A-4D display the percent of CD8+ IFN- secreting cells to specific HIV antigens for four HAART patients receiving HIV vaccination according to the invention.

Figures 5A-5D display plasma viremia in four HAART patients receiving HIV vaccination according to the invention.

Figures 6A-6F display plasma HIV RNA and CD4 T-cell count levels as a function of days on therapy for several patients.

Figures 7A-7F display anti-gp120 and anti-p24 antibody titers for several patients as a function of days post vaccination.

Figures 8A-8F displays intracellular cytokine staining.

Figure 9A-9F display data relating to various HIV antigens.

Figures 10A-10F display stimulation indexes as a function of days post vaccination.

Figures 11A-11-F display stimulation indexes as a function of days post vaccination.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a novel therapeutic modality for treating persons infected with a lymphotropic or immune-destroying retroviral infection. Today, a physician presented with a patient whose immune system is compromised by retroviral infection can select to treat that patient with a host of powerful antiviral agents, including inhibitors of viral proteases and reverse transcriptase. This is known as highly active anti-retroviral therapy (HAART). The conventional HAART protocols are complex and difficult for patients to follow. The drugs also have a number of problematic side effects. In addition, these expensive and complicated treatments fail to eliminate the virus; they merely hold the virus in check. If the patient is non-compliant, the viral count rebounds. Accordingly, for the vast majority of patients, a lifetime of drugs is advised.

The present invention comprises the discovery that after HIV infection, HAART treatment that decreases the viral load can be discontinued using an anti-HIV vaccine that induces an immune response. This response effectively maintains a low titer of virus or controls the viral rebound when the antiretroviral therapy is discontinued,, permitting significant reduction of the patient's dependency on antiretroviral therapy. While some such vaccines have been suggested as useful for seropositive patients (U.S. Patent No. 5,863,542 column 18, lines 60-63), the art has not recognized that administration to seropositive patients receiving anti-viral treatment permits cessation of the anti-viral treatment without virus rebound, with delayed virus rebound, or with decreased post-rebound set point.

The present invention thus provides a method of control of virus rebound in HIV-infected patients after discontinuation of the antiviral therapy. By "control of virus rebound" we mean that after discontinuation of antiviral therapy the viral rebound that usually appears is delayed, the post-rebound set point is decreased, or there is no virus rebound.

Virus rebound appears usually within 1 to 3 weeks after discontinuation of the antiviral therapy. For the purposes of this invention, virus rebound is "delayed" when it appears more than 1 month after discontinuation of the antiviral therapy. Preferably the virus rebound appears more than 2 months and more preferably more than 6 months after discontinuation of the antiviral therapy.

The set point is defined as the plasmatic viral load that is maintained after viral rebound in the absence of antiviral treatment.

Viral rebound can be evaluated by various methods well known in the art. There are a variety of ways to measure viral titer in a patient. A review of the state of the art can be found in the "Report of the NIH to Define Principles of Therapy of HIV Infection" as published in the

Morbidity and Mortality Weekly Reports, April 24, 1998, Vol. 47, No. RR-5, Revised 6/17/98. It is known that HIV replication rates in infected persons can be accurately gauged by measurement of plasma HIV concentrations.

HIV RNA in plasma is contained within circulating virus particles or virions, with each virion containing two copies of HIV genomic RNA. Plasma HIV RNA concentrations can be quantified by target amplification methods (*e.g.*, quantitative 13 RT polymerase chain reaction [RT-PCR], Amplicor HIV Monitor assay, Roche Molecular Systems; or nucleic acid sequence-based amplification, [NASBA®], NucliSens™ HIV-1 QT assay, Organon Teknika) or signal amplification methods (*e.g.*, branched DNA [bDNA], Quantiplex™ HIV RNA bDNA assay, Chiron Diagnostics). The bDNA signal amplification method amplifies the signal obtained from a captured HIV RNA target by using sequential oligonucleotide hybridization steps, whereas the RT-PCR and NASBA® assays use enzymatic methods to amplify the target HIV RNA into measurable amounts of nucleic acid product. Target HIV RNA sequences are quantitated by comparison with internal or external reference standards, depending upon the assay used.

The method of vaccination of the invention is useful for the treatment of HIV-infected patients undergoing an antiretroviral therapy and having a viral load of less than 10,000, preferably less than 5,000, and more preferably less than 1000 viral copies per ml of plasma and a CD4+ T-cell count of above 300 cells/ml, preferably above 500 cells/ml.

By "antiretroviral therapy" or "antiviral therapy" we mean a treatment involving a potent combination of antiviral agents. Antiviral retroviral treatment involves the use of two broad categories of therapeutics. They are reverse transcriptase inhibitors and protease inhibitors. There are two type of reverse transcriptase inhibitors: nucleoside analog reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors. Both types of inhibitors block infection by blocking the activity of the HIV reverse transcriptase, the viral enzyme that translates HIV RNA into DNA that can later be incorporated into the host cell chromosomes. Nucleoside and nucleotide analogs mimic natural nucleotides, molecules that act as the building blocks of DNA and RNA. Both nucleoside and nucleotide analogs must undergo phosphorylation by cellular enzymes to become active; however, nucleotide analogs used are already partially phosphorylated and is one step closer to activation when it enters a cell. Following phosphorylation, the compounds compete with the natural nucleotides for incorporation by HIV's reverse transcriptase enzyme into newly synthesized viral DNA chains, resulting in chain termination. Examples of anti-retroviral nucleoside analogs are: AZT, ddI, ddC, d4T, and 3TC in combination with AZT and Combivir.

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are a structurally and chemically dissimilar group of anti-retrovirals. They are a highly selective inhibitors of HIV-1 reverse transcriptase. At present these compounds do not affect other retroviral reverse transcriptase enzymes such as those from hepatitis viruses, herpes viruses, HIV-2, and mammalian enzyme systems. They are used effectively in triple-therapy regimens. Examples of NNRTIs are Delavirdine and Nevirapine which have been approved for clinical use in combination with nucleoside analogs for treatment of HIV-infected adults who experience clinical or immunologic deterioration. A detailed review can be found in "Non-nucleoside Reverse Transcriptase Inhibitors" A-IDS *Clinical Care (10197) Vol. 9, No. 10, p.* 75.

Proteases inhibitors are compositions that inhibit HIV protease, which is a protease that is virally encoded and necessary for the infection process to proceed.

Clinicians in the United States have a number of clinically effective protease inhibitors to use on HIV infected persons. These include: SAQUINAVIR (Invirase); INDINAVIR (Crixivan); and RITONAVIR (Norvir).

Patients' viral load can be evaluated by various ways. Various methods which can be used have been disclosed above in relation with the virus rebound.

To assess a patient's immune system before antiviral treatment and after treatment as well as to determine if the claimed vaccine regimen is working, it is important to measure CD4+ T-cell counts. A detailed description of this procedure was published by Janet K.A. Nicholson, Ph.D *et* al., "1997 Revised Guidelines for Performing CD4+ T-Cell Determinations in Persons Infected with Human Immunodeficiency Virus (HIV)" in The Morbidity and Mortality Weekly Report, 46(RR-2): [inclusive page numbers], Feb 14, 1997. Centers for Disease Control.

In brief, most laboratories measure absolute CD4+ T-cell levels in whole blood by a multi-platform, three-stage process. The CD4+ T-cell number is the product of three laboratory techniques: the white blood cell (WBC) count; the percentage of WBCs that are lymphocytes (differential); and the percentage of lymphocytes that are CD4+ T-cells. The last stage in the process of measuring the percentage of CD4+ T-lymphocytes in the whole-blood sample is referred to as "immunophenotyping by flow cytometry." Immunophenotyping refers to the detection of antigenic determinants (which are unique to particular cell types) on the surface of WBCs using antigen-specific monoclonal antibodies that have been labeled with a fluorescent dye or fluorochrome (*e.g.*, phycoerythrin [PE] or fluorescein isothiocyanate [FITC]). The fluorochrome-labeled cells are analyzed by using a flow cytometer, which categorizes individual cells according to size, granularity, fluorochrome, and intensity of fluorescence. Size

and granularity, detected by light scattering, characterize the types of WBCs (*i.e.*, granulocytes, monocytes, and lymphocytes). Fluorochrome-labeled antibodies distinguish C7 populations and subpopulations of WBCs. Systems for measuring CD4+T-cells are commercially available. For example Becton Dickenson's FACSCount System automatically measure absolutes CD4+, CD8+, and CD3+ T lymphocytes. It is a self-contained system, incorporating instrument, reagents, and controls.

Patients that can be treated by the method of the invention thus include those newly infected with HIV who have undergone intense anti-retroviral therapy within a few months after infection resulting in a controlled viremia (who can be defined as individuals showing an incomplete Western Blot), as well as chronically-infected individuals undergoing an antiretroviral therapy. By "newly infected" we mean patients who have been infected 90 or fewer days. By "controlled viremia" we mean that the viral load is maintained at a level of less than 10,000 viral copies per ml of plasma.

A preferred population of retrovirally infected persons are those that exhibit CD4+ and CD8+ cell response to HIV antigens, such as those that exhibit proliferative T-cell responses to envelope epitopes, *e.g.*, HIV gp120.

More preferred are those patients that also respond to Gag epitopes, *e.g.*, HIV p24. Typically these patients are identified by measuring the ability of their blood cells to proliferate in responses to highly purified antigen. In brief, peripheral blood monocytes (PBMC) are collected and cultured in the absence of IL-2 and in the presence of 10 μg of highly purified antigen. After four days the cultures are harvested and proliferation is measured by uptake of radioactive thymidine.

An alternative means is to use a skin test. Skin tests involve the detection of a delayed type hypersensitive response (DTH) by means of injecting or scratching antigen beneath the surface of the skin. The reaction is measured by the ability or inability of a patient to exhibit hypersensitive response to an aqueous solution of a gp120 or p24 antigen. Approximately, 1-20 μg is applied. The reaction is determined by measuring wheal sizes from about 24 to about 72 hours after administration of a sample, and more preferably from about 48 hours to about 72 hours after administration of a sample. Preferred wheal sizes for evaluation of the hypersensitivity of a patient range from about 16 mm to about 8 mm, more preferably from about 15 mm to about 9 mm., and even more preferably from about 14 mm to about 10 mm in diameter.

The method comprises administering to an HIV-infected patient as defined above a nucleic acid-based vaccine that enters the cells and intracellularly produces HIV-specific

immunogens for presentation on the cell's MHC class I and MHC class II molecules in an amount sufficient to stimulate efficient HIV-specific CD4+ and CD8+ T-cell responses.

"Efficient CD8+ responses" is referred to as the ability of cytotoxic CD8+ T-cells to recognize and kill cells expressing foreign peptides in the context of a major histocompatibility complex (MHC) class I molecule. CD8+ T-cell responses may be measured, for example, by using tetramer staining of fresh or cultured PBMC, INF-γ Elispot assays, a combination of cell surface phenotyping and cytokine intracellular fluorescence staining intracellular INF-γor using functional cytotoxicity assays, which are well-known to those of skill in the art.

Briefly, for CTL assays, peripheral blood lymphocytes from patients are cultured with HIV peptide epitope at a density of about five million cells/ml. Following three days of culture, the medium is supplemented with human IL-2 at 20 units/ml and the cultures are maintained for four additional days. PBLs are centrifuged over Ficoll-Hypaque and assessed as effector cells in a standard Cr-release assay using U-bottomed microtiter plates containing about 10^4 target cells with varying effector cell concentrations. All cells are assayed twice. Autologous B lymphoblastoid cell lines are used as target cells and are loaded with peptide by incubation overnight during ^{51}Cr labeling. Specific release is calculated in the following manner: (experimental release-spontaneous release)/(maximum release-spontaneous release) x 100. Spontaneous release is generally less than 20% of maximal release with detergent (2% Triton X- 100) in all assays.

"Efficient CD4+ responses" is referred to as the ability of CD4+ T-cells to be stimulated or activated by the vaccine of the invention. CD4+ T cell responses can be measured by various methods well-known in the art.

"Nucleic acid-based vaccine" means DNA and RNA-based vaccines and includes naked nucleic acids and vectored nucleic acids. By "vectored nucleic acid" we mean any kind of viral expression vectors such as DNA and RNA viruses or bacterial vectors such as BCG, salmonella or listeria or lactobacillus that delivers nucleic acid sequences coding for HIV specific immunogen into cells. The vectored nucleic acid corresponds preferably to an attenuated recombinant DNA virus.

"Attenuated recombinant virus" refers to a virus that has been genetically altered by modern molecular biological methods, *e.g.*, restriction endonuclease and ligase treatment, and rendered less virulent than wild type, typically by deletion of specific genes or by serial passage in a non-natural host cell line permissive primary cells or at cold temperatures.

The selection of the virus to be used in the vaccine of the invention is not critical.

Examples of viral expression vectors include adenoviruses as described in M. Eloit *et* al, "Construction of a Defective Adenovirus Vector Expressing the Pseudorabies Virus Glycoprotein gp50 and its Use as a Live Vaccine", J. Gen. Virol., 71(10):2425-2431 (Oct., 1990).), adeno-associated viruses (see, *e.g.*, Samulskl *et* al., J. Virol. 61:3096-3101 (1987); Samulski *et al.*, J. Virol. 63:3822-3828 (1989)), papillomavirus, Epstein Barr virus (EBV) and Rhinoviruses *(see, e.g.,* U.S. Patent No, 5,714,374). Human influenza viruses are also reported to be useful, especially JS CP45 HPIV-3 strain. The viral vector may be derived from herpes simplex virus (HSV) in which, for example, the gene encoding glycoprotein H (gH) has been inactivated or deleted. Other suitable viral vectors include for example retroviruses *(see, e.g.,* Miller, Human Gene Ther. 1:5-14 (1990); Ausubel *et al.*, Current Protocols in Molecular Biology), coksackie viruses, vesicular stomatitis viruses (VSV) and poxviruses.

The poxviruses are preferred for use in this invention. There are a variety of attenuated poxviruses that are available for use as a vaccine against HIV. These include attenuated vaccinia virus, fowlpox virus and canarypox virus. These recombinant virus can be easily constructed. In brief, the basic technique of inserting foreign genes into live infectious poxvirus involves a recombination between poxvirus DNA sequences flanking a foreign genetic element in a donor plasmid and a homologous sequences present in the rescuing poxvirus as described in Piccini *et al.*, Methods in Enzymology 153, 545-563 (1987). More specifically, the recombinant poxviruses are constructed in two steps known in the art and analogous to the methods for creating synthetic recombinants of poxviruses such as the vaccinia virus and avipox virus described in U.S. Pat. Nos. 4,769,330, 4,722,848, 4,603,112, 5,110,587, and 5,174,993, the disclosures of which are incorporated herein by reference.

First, the DNA gene sequence encoding an antigenic sequence such as a known T-cell epitope is selected to be inserted into the virus and is placed into an *E. coli* plasmid construct into which DNA homologous to a section of DNA of the poxvirus has been inserted. Separately, the DNA gene sequence to be inserted is ligated to a promoter. The promoter-gene linkage is positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of poxvirus DNA containing a nonessential locus. The resulting plasmid construct is then amplified by growth within *E. coli* bacteria. Second, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into a cell culture, *e.g.* chick embryo fibroblasts, along with the poxvirus. Recombination between homologous pox DNA in the plasmid and the viral genome gives a poxvirus modified by the presence of foreign DNA sequences in a non-essential region of its genome.

Attenuated recombinant pox viruses are employed in a preferred vaccine. A detailed review of this technology is found in US Patent No. 5,863,542 which is incorporated by reference herein. Representative examples of recombinant pox viruses include recombinant ALVAC and NYVAC. One example of recombinant ALVAC is vCP205. These viruses were deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md., 20852, USA: NYVAC under ATCC accession number VR-2559 on Mar. 6, 1997; vCP205 (ALVAC-MiNI20TMG) under ATCC accession number VR-2557 on Mar. 6, 1997; and, ALVAC under ATCC accession number VR-2547 on Nov. 14, 1996.

NYVAC is a genetically engineered vaccinia virus strain generated by the specific deletion of eighteen open reading frames encoding gene products associated with virulence and host range. NYVAC is highly attenuated by a number of criteria including: i) decreased virulence after intracerebral inoculation in newborn mice, ii) inocuity in genetically (nu^+/nu^+) or chemically (cyclophosphamide) immunocompromised mice, iii) failure to cause disseminated infection in immunocompromised mice, iv) lack of significant induration and ulceration on rabbit skin, v) rapid clearance from the site of inoculation, and vi) greatly reduced replication competency on a number of tissue culture cell lines including those of human origin.

ALVAC is an attenuated canarypox virus-based vector that was a plaque-cloned derivative of the licensed canarypox vaccine, Kanapox (Tartaglia *et al.*, 1992). ALVAC has some general properties which are the same as some general properties of Kanapox.

ALVAC-based recombinant viruses expressing extrinsic immunogens have also been demonstrated efficacious as vaccine vectors. This avipox vector is restricted to avian species for productive replication. On human cell cultures, canarypox virus replication is aborted early in the viral replication cycle prior to viral DNA synthesis. Nevertheless, when engineered to express extrinsic immunogens, authentic expression and processing is observed *in vitro* in mammalian cells and inoculation into numerous mammalian species induces antibody and cellular immune responses to the extrinsic immunogen and confers protection against challenge with the cognate pathogen.

NYVAC and ALVAC have also been recognized as unique among all poxviruses in that the National Institutes of Health ("NIH")(U.S. Public Health Service), Recombinant DNA Advisory Committee (which issues guidelines for the safety containment of genetic material such as viruses and vectors, *i.e.*, guidelines for safety procedures for the use of such viruses and vectors that are based upon the pathogenicity of the particular virus or vector) granted a reduction in physical containment level: from BSL2 to BSL1. No other poxvirus has a BSL1

physical containment level. Even the Copenhagen strain of vaccinia virus (the common smallpox vaccine) has a higher physical containment level; namely, BSL2. Accordingly, the NIH has recognized that NYVAC and ALVAC have a lower pathogenicity than any other poxvirus.

Another attenuated poxvirus of preferred use in the invention is Modified Vaccinia virus Ankara (MVA), which acquired defects in its replication ability in humans as well as most mammalian cells following over 500 serial passages in chicken fibroblasts *(see, e.g.,* Mayr *et al., Infection* 3:6-14 (1975); Carrol, M. and Moss, B. Virology 238:198-211 (1997)). MVA retains its original immunogenicity and its variola-protective effect and no longer has any virulence or contagiousness for animals and humans. As in the case of NYVAC and ALVAC, expression of recombinant protein occurs during an abortive infection of human cells, thus providing a safe, yet effective, delivery system for foreign antigens.

The nucleic acid-based vaccine for use in the present invention further comprises sequences encoding HIV immunogens and intracellularly produces the HIV-specific immunogens. The HIV antigen encoding DNA for insertion into the viral vectors of the invention or for use as naked nucleic acid are any that are known to be effective for protection against a retrovirus. "HIV-specific immunogens" means any HIV protein, fragment, or epitope thereof that is recognized by an immune cell as an epitope of the native protein. HIV-specific immunogens are thus selected from both structural and non-structural proteins. Highly antigenic epitopes for provoking an immune response selective for a specific retroviral pathogen are known.

"Nonstructural viral proteins" are those proteins that are needed for viral production but are not necessarily found as components of the viral particle. They include DNA binding proteins and enzymes that are encoded by viral genes but which are not present in the virions. Proteins are meant to include both the intact proteins and fragments of the proteins or peptides which are recognized by the immune cell as epitopes of the native protein.

"Structural viral proteins" are those proteins that are physically present in the virus. They include the envelope, the capsid proteins, and enzymes that are loaded into the capsid with the genetic material. Because these proteins are exposed to the immune system in high concentrations, they are considered to be the proteins most likely to provide an antigenic and immunogenic response. Proteins are meant to include both the intact proteins and fragments of the proteins or peptides which are recognized by the immune cell as epitopes of the native protein.

The envelope is a preferred source of epitopes and gp 160, 120 and 41 are sources of

immunoprotective proteins. Both B and T cell epitopes have been described in the literature and can be used. Peptides selected from the V3 loop of the HIV envelope proteins are of preferred use. In addition other structural proteins have been reported to be immunoprotective including gp41 and the Gag protein. By "Gag protein" we mean the whole Gag protein as well as proteins derived from Gag such as p17 and p24. Non-structural genes include the *rev, tat, nef, vif*, and *vpr* genes.

For HIV, the nucleic acids include those that can code for at least one of- HIV-I Gag(+ pro)(LAI), gp120(MN or another strain)(+ transmembrane), Nef(BRU)CTL, Pol(IIIB)CTL, ELDKWA or LDKW epitopes, preferably HIV 1 Gag(+ pro)(IIIB), gp120(MN) (+ transmembrane), two (2) Nef(BRU)CTL and three (3) Pol(III)CTL epitopes; or two ELDKWA in gp120 V3 or another region of gp160. The two (2) Nef(BRU)CTL and three (3) Pol(IIIB)CTL epitopes are preferably Nef1, Nef2, Pol1, Pol2 and Pol3. The corresponding sequences are given in U.S. 5,990,091. Furthermore, sequences encoding Tat and/or Rev can advantageously be added. In the above listing, the viral strains from which the antigens are derived are noted parenthetically. The above-defined HIV antigen encoding DNA can be derived from any known HIV strain (HIV1, HIV2, preferably HIV 1), including laboratory strains and primary isolates.

The Pol and Nef epitopes have sequences presented in the following:

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MPLTEEAELE LAENREILKE PVHGVYYDPS KDLIAEIQKQ GQGQWTYQIY QEPFKNLKTG  60
MEWRFDSRLA FHHVARELHP EYFKNCKLMA IFQSSMTKIL EPFRKQNPDI VIYQYMDDLY 120
VGSDLEIGQH RTKIEELRQH LLRWGLTTMV GFPVTPQVPL RPMTYKAAVD LSHFLKEKGG 180
LEGLIHSQRR QDILDLWIYH TQGYFPDWQN YTPGPGVRYP LTFGWCYKLV PMIETVPVKL 240
KPGMDGPKVK QWPLTEEKIK ALVEICTEME KEGKISKIGP                      280
```

where

1-60: CTL epitope Pol-3 (60 aa)

61-86: CTL epitope Nef-2 (26 aa)

89-148: CTL epitope Pol-2 (60 aa)

149-231: CTL epitope Nef-1 (83 aa)

232-280: CTL epitope Pol-1 (49 aa)

Preferred viral vectors according to the invention include ALVAC HIV (vCP1452), which is a recombinant canarypox virus expressing Gag_{LAI}, $Protease_{LAI}$, $Env(120)_{MN}$, $Env(41)_{LAI}$, Nef, and Pol. vCP1452 is described in U.S. Patent Nos. 6,004,777 and 5,990,091. Also useful in the invention is vCP1433, which was deposited with the ATCC in accordance with the Budapest Treaty on March 6, 1997, under accession number VR-2556 and was also

described in U.S. Patent Nos. 6,004,777 and 5,990,091.

Other vectors useful in the invention include those in the table below:

ALVAC-HIV	Inserted HIV genes
vCP125*	$gp160_{MN}$
vCP205**	$gp120_{MN}$ and portion $gp41_{LAI}$, gag_{LAI}, and protease $_{LAI}$
vCP300***	$gp120_{MN}$ and portion $gp41_{LAI}$, gag_{LAI}, and protease $_{LAI}$ and pol CTL domains: 172-219, 325-383, 461-519, nef CTL domains: 66-147, 182-206

*As described in U.S. patent no. 5,766,598.
**ALVAC-MN120TMG deposited on Mar. 6, 1997 as ATCC accession number VR-2557)
***As described in U.S. patent no. 5,863,542.

The administration procedure for recombinant virus and DNA is not critical. Vaccine compositions (*e.g.*, compositions containing the poxvirus recombinants or DNA) can be formulated in accordance with standard techniques well known to those skilled in the pharmaceutical art. Vaccine compositions can comprise one or a plurality of vectors that effect HIV-antigen expression. Such compositions can be administered in dosages and by techniques well known to those skilled in the medical arts taking into consideration such factors as the age, sex, weight, and condition of the particular patient, and the route of administration.

Vaccines may be delivered via a variety of routes of administration including, for example, a parenteral route (intradermal, intramuscular or subcutaneous, transdermal or epidermal). Other routes include oral administration, intranasal, intrarectal and intravaginal routes. Examples of vaccine compositions of use for the invention include liquid preparations, for orifice, *e.g.*, oral, nasal, anal, vaginal, etc. administration, such as suspensions, syrups or elixirs; and, preparations for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (*e.g.*, injectable administration) such as sterile suspensions or emulsions. In such vaccines the naked or vectored nucleic acid may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, Tris buffer or the like. The vaccine of the invention may also comprise an adjuvant. Any adjuvant administrable to humans can be used. Adjuvants useful in the invention include alum, calcium phosphate and, preferably PCPP (poly dicarboxylatopheoxylphosphazene), a synthetic hydrogel polymer developed for its adjuvant properties.

A viral vector- based vaccine can be administered at about 10^3-10^8 TCID50/dose or 10^4

to 10^9 pfu per dose. For example, ALVAC-HIV vaccine is inoculated, more than once, by the intramuscular route at a dose of about 10^8 pfu per inoculation, for a patient of 170 pounds. The vaccine may be delivered in a physiologically compatible solution such as sterile 0.4% NaCl in a volume of, *e.g.*, one ml. The vaccine of the invention is administered several times. Intervals between administrations and number of administration depend of the immune response of the patient. Vaccine doses have to be administered as long as it is necessary to re-induce the immune system. Actual dosages of such a vaccine can be readily determined by one of ordinary skill in the field of vaccine technology.

As an alternative to a viral vaccine, DNA may also be directly introduced into the cells of a patient. This embodiment is defined in the present invention as naked-DNA vaccine. This expression (*i.e.*, naked-DNA vaccine) thus encompasses naked DNA *per se*, including virus like particles, as well as formulated DNA-based vaccines as disclosed below. This approach is described, for instance, in Wolff *et. al.*, *Science* 247:1465 (1990) as well as U.S. Patent Nos. 5,580,859; 5,589,466; 5,804,566; 5,739,118; 5,736,524; 5,679,647; and WO 98/04720. Examples of DNA-based delivery technologies include, "naked DNA," facilitated (bupivicaine, polymers, peptide-mediated, adjuvants) delivery, and cationic lipid complexes or liposomes and microspheres. Alternatively, the nucleic acids can be administered using ballistic delivery as described, for instance, in U.S. Patent No. 5,204,253 or pressure (see, *e.g.*, U.S. Patent No. 5,922,687). Using this technique, particles comprised solely of DNA are administered. In a further alternative embodiment, DNA can be adhered to particles, such as gold particles. As is well known in the art, a large number of factors can influence the efficiency of expression of antigen genes and/or the immunogenicity of DNA vaccines. Examples of such factors include the reproducibility of inoculation, construction of the plasmid vector, choice of the promoter used to drive antigen gene expression and stability of the inserted gene in the plasmid. Any of the conventional vectors used for expression in eukaryotic cells may be used for directly introducing DNA into tissue. Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, *e.g.*, CMV vectors. Other exemplary eukaryotic vectors include pMSG, pAV009/A+, pMTO10/A+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 later promoter, metallothionein promoter, human cytomegalovirus promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Therapeutic quantities of plasmid DNA can be produced, for example, by fermentation

in *E. coli* followed by purification. Aliquots from the working cell bank are used to inoculate growth medium and grown to saturation in shaker flasks or a bioreactor according to well known techniques. Plasmid DNA can be purified using standard bioseparation technologies such as solid phase anion-exchange resins supplied by QIAGEN, Inc. (Valencia, California). If required, supercoiled DNA can be isolated from the open circular and linear forms using gel electrophoresis or other methods.

Purified plasmid DNA can be prepared for injection using a variety of formulations. The simplest of these is reconstitution of lyophilized DNA in sterile phosphate-buffer saline (PBS). This approach, known as "naked DNA," is currently being used for intramuscular (IM) administration in clinical trials.

To maximize the immunotherapeutic effects of minigene DNA vaccines, an alternative method for formulating purified plasmid DNA may be desirable. A variety of methods have been described, and new techniques may become available. Cationic lipids can also be used in the formulation (*e.g.*, as described by WO 93/24640; Mannino & Gould-Fogen'te, *BioTechniques* 6(7): 682 (1988); U.S. Pat No. 5,279,833; WO 91/06309; and Felper, *et al., Proc. Nat'l Acad. Sci. USA* 84:7413 (1987). In addition, glycolipids, fusogenic liposomes, peptides targeting sequences and compounds referred to collectively as protective, interactive, non-condensing compounds could also be complexed to purified plasmid DNA to influence variables such as stability, intramuscular dispersion, or trafficking to specific organs or cell types. DNA expression vectors for direct introduction of DNA into the patient tissue can additionally be complexed with other components such as peptides, polypeptides, lipopeptides, carbohydrates, microspheres, immunostimulants and adjuvants. Expression vectors can also be complexed to particles or beads that can be administered to an individual, for example, using a vaccine gun.

The expression vectors are administered by methods well known in the art as described, for example, in Donnelly *et al.* (*Ann. Rev. Immunol.* 15:617-648 (1997)); Felgner *et al.* (U.S. Patent No. 5,580,859, issued December 3, 1996); Felgner (U.S. Patent No. 5,703,055, issued December 30, 1997); and Carson *et al.* (U.S. Patent No. 5,679,647, issued October 21, 1997), each of which is incorporated herein by reference. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the expression vector.

For example, naked DNA or polynucleotide in an aqueous carrier can be injected into tissue, such as muscle or skin, in amounts of from 10 l per site to about 1 ml per site. The concentration of polynucleotide in the formulation is from about 0.1 μg/ml to about 20 mg/ml.

Actual dosages of the vaccine can be readily determined by one of ordinary skill in the field of vaccine technology

The expression vectors of use for the invention can be delivered to the interstitial spaces of tissues of an animal body (Felgner *et al.*, U.S. Patent Nos. 5,580,859 and 5,703,055). Administration of expression vectors of the invention to muscle is a particularly effective method of administration, including intradermal and subcutaneous injections and transdermal administration. Transdermal administration, such as by ionophoresis, is also an effective method to deliver expression vectors of the invention to muscle. Epidermal administration of expression vectors of the invention can also be employed. Epidermal administration involves mechanically or chemically irritating the outermost layer of epidermis to stimulate an immune response to the irritant (Carson *et al.*, U.S. Patent No. 5,679,647). The vaccines can also be formulated for administration via the nasal passages. Formulations suitable for nasal administration, wherein the carrier is a solid, include a coarse powder having a particle size, for example, in the range of about 10 to about 500 microns which is administered in the manner in which snuff is taken, *i.e.*, by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations wherein the carrier is a liquid for administration as, for example, nasal spray, nasal drops, or by aerosol administration by nebulizer, include aqueous or oily solutions of the active ingredient. For further discussions of nasal administration of AIDS-related vaccines, references are made to the following patents, US 5,846,978, 5,663,169, 5,578,597, 5,502,060, 5,476,874, 5,413,999, 5,308,854, 5,192,668, and 5,187,074.

The vaccines can be incorporated, if desired, into liposomes, microspheres or other polymer matrices (Feigner *et al.*, U.S. Patent No. 5,703,055; Gregoriadis, *Liposome Technology*, Vols. I to III (2nd ed. 1993), each of which is incorporated herein by reference). Liposomes, for example, which consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

Liposome carriers may serve to target a particular tissue or infected cells, as well as increase the half-life of the vaccine. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the vaccine to be delivered is incorporated as part of a liposome, alone or in conjunction with a targeting molecule which binds to, *e.g.*, a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes either filled or decorated with a

desired immunogen of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the immunogen(s).

Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol such as cholesterol. The selection of lipids is generally guided by consideration of, *e.g.*, liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, *e.g.*, Szoka, *et al.*, *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

Vaccines for use in the present invention can be administered alone or can advantageously be combined with an immunostimulating composition and/or another anti-HIV vaccine.

By "combined" we mean a simultaneous or a sequential administration (*e.g.*, prime-boost) of a vaccine and an immunostimulating composition and/or of another anti-HIV vaccine.

Vaccines for use in the invention can advantageously be combined with immunostimulatory or co-stimulatory molecules such as for example cytokines, interleukin 2 or CD40 ligand, which are used in an amount that is sufficient to potentiate the T-cell responses, in particular CD8+ responses. These immunostimulating compounds are used according to the recommendations of the manufacturer. Such compounds may be present as such or in the form of a recombinant virus expressing the same.

Vaccines for use in the invention can advantageously be combined with another anti-HIV vaccine. Such anti-HIV vaccine can be different from the first vaccine (for example, naked nucleic acid-based vaccine can be combined with a viral vector-based vaccine, naked DNA followed by a HIV immunogen-encoding poxvirus, or an HIV-immunogen encoding attenuated vaccinia virus followed by a HIV immunogen-encoding avipox virus), or can be a vaccine comprising a soluble antigen of HIV. Any soluble HIV antigen that is known to be an effective antigen for protection against HIV can be used. According to a preferred embodiment, the soluble antigen corresponds to the gp160 HIV-1 envelope glycoprotein and, in particular, the gp160MN/LAI-2, corresponding to an envelope glycoprotein from HIV-1 virus expressed by vaccinia virus VV.TG.9150 on BHK_1 cells wherein the gp120 portion is derived from HIV_{MN} and the gp41 transmembrane portion from HIV_{LAI}. Actual dosages of the soluble antigen can be readily determined by one of ordinary skill in the field of vaccine technology

According to a preferred embodiment, the vaccine comprises a nucleic acid vector (*e.g.*, a viral vector) comprising genes encoding and expressing a plurality of HIV antigens and is co-administered with an HIV antigen. In a most preferred embodiment, a vector comprising the

ALVAC canarypox vector expressing the HIV Gag, Protease, Env(120), Env (41), Nef, and Pol antigens is co-administered with the gp160 HIV-1 envelope glycoprotein.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

EXAMPLES

HAART Therapy

Ongoing HAART clinical trials at the Aaron Diamond AIDS Research Center are summarized in Table 1:

Table 1

Clinical Trials at the ADARC of the Rockefeller University

Study Identifier	Treatment Regimen	# active/# recruited	Study Population	Duration of therapy (mos.)
MMA-160	AZT/3TC/RIT	8/12	Newly infected	26-32
MMA-167	AZT/3TC/IND	11/12	Newly infected	19-25
MMA-174	AZT/3TC/NLF	8/12	Infection>90d	27
MMA-183	RIT/SAQ	10/12	Infection>90d	21
MMA-197	AZT/3TC/	12/14	Newly infected	13-20
	RIT/SAQ	10/13	Inf.>90d	19
MMA-227	AZT/3TC/1592/	12/13	Newly infected	1-12
	GW141	11/12	Inf.>90d	7-12

RIT=ritonavir IND=indinavir NLF=nelfinavir SAQ=saquinavir 1592=Abacavir GW141=Vertex 478 (Protease inhibitor)

The clinical program divided study subjects into two groups, those newly infected and those infected for greater than 90 days on entry into the screening phase.

New infections were diagnosed on the basis of a positive plasma HIV-1 RNA in the setting of one of the following three criteria: absence of HIV-antibody by ELISA, progression of the antibody response as determined by the appearance of at least two new bands on Western

blot and a clinical syndrome consistent with acute infection within 90 days of screening, and a documented negative test within the previous 120 days.

Participants in these clinical trials were generally followed weekly for four weeks, bi-weekly for two months, then monthly to assess for both safety and efficacy. Routine laboratory determinations include plasma HIV-RNA levels using either bDNA signal amplification or PCR technology, safety laboratory studies including routine hematology and chemistry, and assessments of immunologic status including a variety of cell surface markers used to define naïve and memory cell subsets.

Representative longitudinal plasma HIV-RNA and CD4 cell data of a chronically infected cohort participating in study MMA-197 is shown in Figure 1. As depicted in Fig. 1, suppression of virus replication is accompanied by a 2 log drop in HIV RNA during the early weeks. Further suppression of the productive infection of new susceptible cells results in a continued drop in the plasma HIV-1 RNA reflecting the loss of cells continuing to produce non-infectious virus particles. The antiviral effect is dramatic and results in a nearly 4 log reduction as the nadir is reached at week 24.

The impact of complete suppression of virus replication can be viewed in a somewhat different way in Figure 2. As the weeks of therapy progress the level of HIV-RNA measured in this group of treated subjects becomes increasingly difficult to detect. By the end of 48 weeks, all of the subjects treated with this four drug combination met the goal of "undetectability." These results suggest that the total pool of infected cells still producing particles at this time point has fallen to a very low level.

Lymphoid tissue was obtained from patients participating in these studies after a minimum of 12 months of HAART therapy. Gastrointestinal-associated-lymphoid tissue (GALT) was obtained in the majority of subjects. Biopsies were graded on a scale of 1 to 4; 1=scattered lymphoid cells, 2=small lymphoid aggregate, 3=large well defined aggregate, 4=germinal center present. Individuals also agreed to undergo tonsillar biopsy or lymph node biopsy. Eight subjects underwent gastrointestinal biopsy. In 4 in whom follicles were present, no trapped virus was detected. In all 8, a limited number of tissue sections examined did not reveal RNA expressing cells. With extensive sampling of the biopsied material from subject 9 the rare expression of HIV-specific RNA could be detected in rectal tissue, tonsil, and cervical node. Germinal centers were free of trapped virus and the rare RNA-positive cell had relatively few grains (7 to 37) compared to untreated controls in which the grain number was too numerous to count.

To maximize the detection of potentially infectious virus, we performed co-cultures of

mononuclear cells (MC) from blood after depletion of CD8+ T-cells to remove potential inhibitory soluble factors and stimulated the MC with PHA.

Using the method of Saksela and Vesanen, nested PCR for both multiply-spliced (MS) and unspliced (U.S.) -HIV-mRNA and proviral DNA were performed on MC from blood and lymphoid tissue. The results of these studies in the peripheral blood from subjects participating in study MMA-160 is summarized in Table 2.

Table 2

Blood

Subject	Duration of Therapy (months)	PHA Stimulated Culture $TCID_{50}/10^6$ CD8-CD4)	RNA PCR multiply spliced copies/mg	RNA PCR Unspliced copies/mg	DNA PCR (copies/10^6 PBMC)
2	24	<0.1	<50	372	353
3	23	<0.1	<50	459	503
5	22	>0.1	<50	1921	528
6	20	>0.1	<50	295	254
7	20	<0.1	<50	167	167
8	19	<0.1	<50	284	284
9	19	>0.1	<50	112	112
11	18	>0.1	312	1521	1753

Viral load of CD8+ T cell-depleted PHA-stimulated co-cultures after 19 to 24 months of therapy were less than 0.1 $TCID_{50}/10^6$ CD4 in Subjects 2, 3, 7, and 8. Cultures were strongly positive in subjects 6, 9 and 11 and borderline positive in Subject 5. Quantitative PCR detected both MS and US-mRNA in PBMC from subject 11.

US-mRNA was detected in PBMC from subjects 2,3, 5 and 6. PBMC from subjects 7, 8, and 9 did not reveal detectable mRNA.

Culture and quantitative PCR results for GALT and other lymphoid tissues obtained early in the second year of therapy in the same cohort of early treated subjects are shown in Table 3. These are compared to a control with high levels of virus replication in blood and lymphatic tissue.

Table 3

GALT

Subject	Duration of Therapy (months)	Site of biopsy	Culture ($TCID_{50}/10^6$ PBMC)	RNA PCR multiply spliced copies/mg mRNA	RNA PCR unspliced copies/mg mRNA	DNA PCR (copies/10^6 PBMC)
Positive control	N/A	desc. colon	1	439	256,062	5,346

Subject	Duration of Therapy (months)	Site of biopsy	Culture ($TCID_{50}/10^6$ PBMC)	RNA PCR multiply spliced copies/mg mRNA	RNA PCR unspliced copies/mg mRNA	DNA PCR (copies/10^6 PBMC)
		sigmoid		375	194,338	2,230
		rectum		351	170,851	3,624
2	16	desc. colon	<0.25	<50	<50	150
		sigmoid		<50	<50	ND
		rectum		<50	<50	121
3	17	desc. colon	<0.1	<50	331	10
		sigmoid		<50	<50	99
		rectum		<50	<50	117
5	16	desc. colon	<1.0	<50	155	<10
		sigmoid		<50	101	ND
		rectum		<50	217	58
6	13	desc. colon	<1.0	<50	102	ND
		sigmoid		<50	<50	83
		rectum		<50	<50	<10
7	15	desc. colon	<0.1	<50	110	<10
		sigmoid		<50	<50	23
		rectum		<50	<50	<10
8	14	desc. colon	<0.1	<50	101	260
		sigmoid		<50	<50	68
		rectum		<50	<50	540
9	12	desc. colon	<0.1	<50	<50	<10
		sigmoid		<50	411	<10
		rectum		<50	<50	<10
9	15	tonsil 1	<0.1	<50	345	76
		tonsil 2		<50	245	56
		lymph node 1	<0.1	<50	987	28
		lymph node 2		<50	<50	<10
		sigmoid 1	<0.1	<50	<50	<10

Subject	Duration of Therapy (months)	Site of biopsy	Culture ($TCID_{50}/10^6$ PBMC)	RNA PCR multiply spliced copies/mg mRNA	RNA PCR unspliced copies/mg mRNA	DNA PCR (copies/10^6 PBMC)
		sigmoid 2		<50	454	14
		rectum		<50	<50	38
11	13	desc. colon	<0.1	<50	<50	150
		sigmoid		<50	<50	<10
		rectum		<50	<50	207

Studies performed on GALT during months 12 to 17 are similarly presented. MC co-cultures were routinely below the level of detection as was the level of MS-mRNA. US-mRNA was detected in very low levels in all subjects except 2 and 11. Proviral DNA was routinely detected in the MC of all subjects. At the month 15 visit subject 9 underwent tonsil and cervical lymph node biopsy. Similar results are observed in these samples; no culturable virus, undetectable MS-mRNA, low level US-RNA expression, and low copy number of proviral DNA.

A lumbar puncture was performed in subjects 3 and 9 at months 24 and 15, respectively. In both, the fluid was acellular and had less than 25 HIV-RNA copies/ml as determined by ultra-sensitive RNA PCR (Roche).

Analysis of semen concurrent with lymphoid tissue biopsy revealed mononuclear cells (MC) with undetectable levels of both multiply-spliced and unspliced HIV-mRNA. Proviral DNA was detected at low levels, between 10 and 100 copies/10^6 MC in all but one subject (#9).

During various clinical studies intensive virologic measurement were performed in early infected HAART treated subjects. New infections were diagnosed on the basis of a positive plasma HIV-1 RNA in the setting of one of the following three criteria: absence of HIV-antibody by ELISA, progression of the antibody response as determined by the appearance of at least two new bands on Western blot and a clinical syndrome consistent with acute infection within 90 days of screening, and a documented negative test within the previous 120 days.

The results of these studies suggest that as these newly infected subjects reach the second year of therapy, there exists a minimal level of HIV-1 expression. It cannot be determined that HIV expression necessarily translates into ongoing rounds of infection of susceptible cells, but may represent stochastic activation of the latently infected population that is controlled by the presence of the antiviral regimen.

The reduction in total body virus burden has a significant effect on both CTL precursor

frequencies and antibody levels to Gag and Env in this cohort of newly infected subjects. As seen in Figure 3, subjects 3 and 8, levels of CTLp drop with time as HIV replication is inhibited. Similar results are seen in similarly treated subjects in both newly infected and chronically infected cohorts.

Similarly, persistent control of virus replication results in significant reductions in HIV-specific antibodies to Env (gp120) and Gag (p24). This has been observed, however, only in the newly infected and not the chronically infected treatment group.

Based on the low level of HIV-specific immune responses as a consequence of effective antiviral therapy and the small pool of latently infected cells harboring potentially infectious virus, we concluded that stimulation of HIV specific immune response would be desirable prior to discontinuation of antiviral therapy. We believed that based on the results from studies of newly infected subjects and long-term non-progressors with minimal virus activity, CD4+ T-cell and CTL activities are critical immunologic control factors. Other data suggested to us that high levels of neutralizing antibodies are associated with lack of disease progression.

Our vaccine strategy is based on the concept that both humoral and cell-mediated immune responses can be stimulated by stimulating the immune system with live recombinant vectors expressing various HIV-1 antigens and with soluble recombinant proteins as discussed above.

HIV Vaccine Research Design and Methods

Subjects already participating in ongoing HAART clinical trials conducted by the clinical arm of the Aaron Diamond AIDS Research Center were eligible for participation in this study.

A. Pre-entry Virologic Evaluation

HIV-infected subjects participating in one of the HAART clinical trials at ADARC (newly infected) underwent extensive virologic evaluation after a minimum of two years of therapy.

Blood, lymphoid tissue including tonsil and/or lymph node(Study #MMA-189), semen (#MMA-205), and CSF (#MMA-203) were collected on all consenting subjects. Participation required informed consent by signature for each procedure listed above. No subject was excluded from participation in this vaccine study based on participation in these other studies of tissue and body fluids (see inclusion/exclusion criteria, below).

Blood was processed as follows; plasma was separated by centrifugation and stored at -

70°C for subsequent studies as well as ultra-sensitive HIV-RNA determination using a modification of the Roche Amplicor assay.[43] This assay was the most sensitive and reproducible assay available to determine levels of HIV-1 RNA in plasma. Peripheral blood mononuclear cells (PBMC) are isolated by Ficoll-Hypaque gradient using standard techniques. Aliquots of a minimum of 10^7 cells were prepared and stored at -150°C for future use. Cells were CD8 depleted using magnetized-antibody-coated polystyrene beads (Dynal). $1\text{-}2x10^7$ CD8-depleted MC were stimulated with PHA and irradiated feeder cells and co-cultured in IL-2 containing medium with HIV-negative donor CD4+ T-cells. Cultures were maintained for three weeks and culture supernatants assayed weekly for levels of p24. A positive culture requires a p24 concentration of at least 100 pg/ml in the culture supernatant.

As the lymphoid system is the preferred site of virus replication in an infected host, a comprehensive surgical program was established at Rockefeller University Hospital to meet the specific needs of the ADARC clinical program. A general surgeon to perform inguinal lymph node biopsy and an otolaryngologist (ENT) to perform either cervical node or tonsillar biopsy were recruited. A board-eligible gastroenterologist obtained gastrointestinal-associated lymphoid tissue (GALT). These procedures were done under separate protocols MMA-189 and ATA-207.

Consenting subjects were well-known to the clinical staff, but screening for coagulopathy with measurements of prothrombin time (PT) and partial thromboplastin time (PTT) was included prior to procedure. A careful surgical history was also required to screen for rarer causes of hemostatic dysfunction. Biopsies were performed using local anesthesia without the need for conscious sedation. Lymphoid tissue was divided into three sections, a portion immediately frozen in liquid nitrogen for PCR analysis, a portion formalin-fixed and subsequently paraffin embedded for *in situ* hybridization and immunohistochemistry, and a portion transported in culture medium from which MC were mechanically disrupted and cultured using standard co-culture techniques.

All culture supernatants positive for HIV-RNA were analyzed for the presence of either genotypic or phenotypic resistant virus. Similarly, all plasma samples with HIV-1 RNA above 500 copies/ml were used for RT-PCR, although the limitations of this assay at low copy number was well appreciated.

Semi-quantitative PCR for multiply-spliced (MS) and unspliced (US)-mRNA as well as proviral DNA were performed on PBMC and MC from semen, cervical lavage, and lymphoid tissue with a modified technique of Vesanen and Saksela.[44-46]

Finally, paraffin embedded sections of lymphoid tissue were subject to in-situ hybridization pursuant to published techniques.[47,48]

Subjects eligible for vaccination had to meet the following virologic criteria:

1. Undetectable levels of MS-mRNA in blood and/or tissue
2. Rare to no HIV expressing cells by in-situ hybridization (tissue sampling is optional)
3. Viral cultures from blood and/or tissue either negative for culturable virus or yielding drug-sensitive virus by genotype and phenotype

Subjects failing to meet these virologic criteria could be re-evaluated at 6 month intervals.

B. HIV-Specific Immunologic Evaluation

Simultaneous immunologic investigations were performed after two years of therapy to determine eligibility for vaccination.

Direct CTL effector activity was measured from freshly isolated PBMC using autologous B-lymphoblastoid cell targets infected with recombinant vaccinia virus expressing HIV-1 specific genes (gag, pol, env, nef).[4]

HIV-specific CTL precursor frequencies (CTLp) were similarly performed in selected subjects.[49] Patient PBMC were seeded at varying concentrations in 200 µl of IL-2-containing medium in 24 replicate-wells of a 96-well tissue culture plate. Irradiated donor PBMC and anti-CD-3 antibody were added to each well and incubated at 37°C for 14 days. Wells were split into four and assayed for the ability to lyse an autologous chromium-labeled B-lymphoblastoid cell line infected with a vaccinia-virus expressing HIV-1 env, gag, pol, and nef genes as well as an antigen negative control. CTLp with a given specificity were determined by plotting the log of the fraction of negative wells (less than 3 S.D. above the mean for the 24 control wells or below 10% specific lysis) versus the number of input cells.[4]

Patients with detectable fresh CTL activity above 30% specific lysis to one or more antigens at an effector to target ratio of 25:1 were not eligible for participation in the vaccination protocol. Subjects with levels of CTL precursors above 1 in 100,000 to one or more specific antigens including Env, Gag, Pol, or Nef were similarly excluded.

C. Inclusion Criteria

The following criteria were used to select patients for the vaccination study:

- HIV infected subjects with at least 2 years of combination antiretroviral therapy
- Plasma HIV-RNA < 25 copies/ml

- Absent Multiply Spliced (MS) RNA determinations in peripheral blood
- Qualitative CD4 cell co-culture either negative or positive for wild-type virus (as determined by genotype) from blood
- Ability to give informed consent
- Age greater than 18
- There were no CD4+ T cell count entry criteria

D. Optional Entry Criteria

In subjects agreeing to tissue biopsy or body fluid collection (genital secretions, CSF), after 24 months of therapy, the following virologic criteria had to be met:

- Absent Multiply Spliced (MS) RNA determinations
- Rare to no HIV expressing cells in tissue by in situ hybridization
- Qualitative CD4 cell co-culture either negative or positive for wild-type virus (as determined by genotype)

E. Exclusion Criteria

The following criteria were used to exclude patients from the vaccination study:

- Evidence of cellular immune responses to HIV-1 defined by:
  - Fresh CTL activity above 30% specific lysis to one or more antigens at an effector to target ratio of 25:1
  - CTLp above 1 in 100,000 to one or more specific HIV antigens
- Pregnancy
- Breast feeding
- Clear evidence of HIV replication in the presence of combination drug therapy as evidence by one of the following: Plasma HIV-RNA above the level of detection on 2 consecutive tests more than 2 weeks apart, evidence of multiply-spliced (MS) HIV-RNA species in peripheral blood, or the presence of culturable virus from blood that harbors genotype consistent with drug resistance to one or more of the current antiretroviral agents included in the subject's treatment regimen.
- If tissue was obtained after 24 months of therapy then patients were excluded if there was MS-HIV-RNA species demonstrated by PCR or CD4-co-culture yielded drug resistant virus (based on genotype). In addition, the presence of trapped virus in the follicular dendritic cell network as seen by in situ hybridization will resulted in exclusion.

- Laboratory data:

  Hemoglobin <9.0 g/l

  Absolute granulocyte less than 1000 cell/mm^3

  Platelets less than 75,000/mm3

  ALT and/or AST greater than 2.5 times the upper limit of the normal range (ULN)

  Amylase above 1.5 times the ULN

  Creatinine above 1.5

  Bilirubin (direct) above 1.5

- Allergy to eggs and/or neomycin

F. **Screening Procedures**

Screening was done within 60 days of receiving the first dose of vCP1452 and rgp160.

Screening procedures included:

Complete history and physical examination

Laboratory assessments for safety at baseline:

Hematology

CBC with platelets and differential

Chemistry

Electrolytes

BUN/creatinine

Amylase

AST, ALT, alkaline phosphatase, bilirubin

Albumin, total protein

Calcium, magnesium, phosphate

Urinalysis

dipstick

microscopic analysis

Other

urine pregnancy test (prior to each vaccination)

Virology*

HIV-RNA (RT-PCR)

PBMC RT-PCR for HIV-RNA

Proviral DNA (integrated and un-integrated)

CD4+ lymphocyte co-culture

Immunology*

CTLe (bulk)

CTLp

CTLe (tetramers)

HIV-specific proliferation assays to HIV antigens

HIV-specific antibody levels (p24 and gp120)

*Blood was drawn at 2 weeks, then monthly for virology and immunology. Assays other than HIV-RNA were performed at the discretion of the investigators, but no less than every three months. HIV-RNA was performed at each visit.

Pregnancy test (serum) when applicable

Virology studies

Immunology studies

G. Description of Vaccines:

ALVAC HIV (vCP1452) is a recombinant canarypox virus expressing the gag_{LAI}, $protease_{LAI}$, $env(120)_{MN}$, $env(41)_{LAI}$, nef, and pol genes. VCP1452 is described in U.S. Patent Nos. 6,004,777 and 5,990,091.

vCP1452 is modified to include 2 vaccinia virus coding sequences to enhance expression in mammalian cells. The pol and nef sequences are scrambled such that no functional proteins can be expressed., Approximately 10^7 $TCID_{50}$ in 1.0 ml were given with each dose.

Recombinant gp160MN/LAI-2 is an envelope glycoprotein from HIV-1 virus expressed by vaccinia virus VV.TG.9150 on BHK_1 cells. The gp120 portion is derived from HIV_{MN} and the transmembrane gp41 portion from HIV_{LAI}. The adjuvant, PCPP, is a synthetic soluble polymer developed for its adjuvant properties. The vaccine contained 50μg of recombinant gp160 in 500 μg PCPP (1.0 ml).

The vaccines being used in this study as well as the adjuvant are novel.

H. Schedule for vaccination

12 subjects meeting inclusion criteria were treated as follows:

ALVAC-HIV (vCP1452) and recombinant soluble gp160MN/LAI-2 were administered intramuscularly on days 0, 30, 90, 180. For ALVAC HIV (vCP1452), each vaccination dose was 1.0 mli.m. [approximately 10^7 $TCID_{50}$]; for gp160 MN/LAI-2, each vaccination dose was

50μg in 500μg PCPP (1.0 ml).

Patients remained in the clinic area for 30 minutes after each and every vaccination. All subjects were contacted by telephone within 72 hours of each vaccination to document any adverse events. These interviews were recorded in the patient's record.

I. Patient Visits and Procedures Other than Vaccination Schedule (as above)

On day 0 subjects received:

Diary to record adverse events to be given to subjects

First dose of vaccines as outlined in protocol

On day 2 the following were performed:

Complete history and physical

Safety laboratory assessments (described above)

Urine pregnancy test (when applicable)

Virologic assessments

Immunologic assessments

3. Clarification

Screening procedures were performed within 60 days of receiving the vaccines (day 0) in addition to an additional assessment at day -2.

Week 1 Interval history and physical

Review of patient diary

Safety laboratory assessments (described above)

Week 2 Interval history and physical

Review of patient diary

Safety laboratory assessments (described above)

Virologic assessments

Immunologic assessments

Months 1-8 Interval history and physical

Review of patient diary

Safety laboratory assessments (described above)

Urine pregnancy test (when applicable)

Virologic assessments

Immunologic assessments

Post-vaccine* Interval history and physical

(1 week) Review of patient diary

Safety laboratory assessments

Virologic assessments

*within 7 days of receiving vaccine on day 0, 30, 60, 120

J. Safety Considerations

12 subjects were vaccinated and the safety and immunogenicity assessed as outlined above.

Many people have been given vaccines similar to the gp160 portion of this study without significant side effects.

The ALVAC portion given with the gp160 portion together has caused at least one of the following side effects in at least 75% of the subjects: pain and redness at the site of injection, weakness, muscle aches, joint aches, headache, and fever above 38°C. ALVAC is an avian virus (canarypox) that cannot replicate in man and therefore undergoes only one abortive cycle of replication. Over 1800 subjects received an ALVAC construct without significant serious adverse events. Additionally, over 700 subjects received ALVAC/soluble Env vaccine regimens with no severe reactions (unpublished data).

Participants were vaccinated as outpatients at the clinical site, Rockefeller University Hospital. This General Clinical research Center is staffed with highly skilled and experienced personnel. Emergency medications and equipment, known commonly as a "crash cart" were available for use in the clinic area.

Participants were monitored closely for 30 minutes post immunization for evidence of adverse events. Participants were given diaries to record any adverse event. These diaries were reviewed at each visit.

Any Grade 3 or 4 toxicity that could be definitively determined to be related to the vaccine must result in patient discontinuation.

One death has been recorded in one trial but not deemed related to the vaccine

K. Immunogenicity

Antibody titers, proliferative responses and CTL activity to HIV specific antigens were measured at baseline and post-vaccination as indicated using standard techniques. Blood was drawn and cells and plasma stored for immunologic and virologic studies on days 0, 15, 30, 60, 90 120, 180, and 210 and the above assays performed. Criteria for response included: a two-fold increase in antibody titer to env and/or gag, a measurable increase in level and/or broadening of detectable fresh CTL activity and/or CTLp, and a three fold increase in

proliferation index to HIV specific antigens measured *in vitro*.

Responders: Subjects demonstrating an immune response to the vaccines without significant adverse events, that is, no Grade 3 or 4 nor significant local reactions, were offered the opportunity to participate in an extension that provides for vaccination every three months with identical follow-up, that is, observation in clinic for 30 minutes, telephone follow-up within 72 hours, diary cards to record temperature and adverse events, clinic visits 2 weeks after vaccination, and careful virologic monitoring, all as described above.

Failures: Subjects failing to demonstrate a response to the vaccines after day 180 as defined by the immunogenicity criteria outlined above were asked to receive vaccination with 0.5 ml tetanus toxoid to test for the ability to respond to recall antigens. Blood was drawn 1 month later to assess for an immune response (serology). Virus activity was carefully monitored with a clinic visit and virologic evaluation 2 weeks and 1 month post vaccine in addition to regularly scheduled clinic visits.

L. Extension

Individuals who are considered responders on the basis of a documented immune response, either humoral or cell mediated continued to be vaccinated with ALVAC vCP1452 and recombinant gp160 with PCPP every three months for a total of 12 months.

M. Biostatistics

Immunogenicity was determined by baseline and post vaccination measurement of: CTL activity using bulk CTL assays, CTLp frequencies, CTLe frequencies by tetramers if available, proliferation to HIV-specific antigens *in vitro*, and levels of HIV specific antibodies to gp120 and p24.

Subjects were followed with virologic assessments simultaneously. Increases in immunologic parameters listed above without evidence of increases in plasma HIV-RNA, PBMC-associated multiply-spliced and unspliced HIV-RNA, or abrupt changes in levels of CD4+cell-associated proviral DNA were interpreted as being the result of exposure to vaccine antigens as opposed to the result of activation of virus replication.

N. Human Subjects

1. Characterization of the study population

Patients over the age of 18 with documented HIV infection and treated on one of the Aaron Diamond AIDS Research Center protocols were invited to participate. All subjects met the virologic and immunologic criteria outlined above to participate. As the effect on fetuses

and newborns of the vaccines used in this study, ALVAC and gp160, are unknown, all participants agreed to use double barrier contraception to prevent pregnancy.

2. Source of research material

After signing consent forms patients were enrolled. All antiretroviral medications were discontinued throughout the course of this study up to day 240 following initial vaccination with the ALVAC HIV (vCP1452) and gp160.

Subjects were allowed, if desired, to participate in this vaccine protocol without consenting to collection of tissue and or fluid other than blood. These were optional procedures and serve to establish the absence of virus replication as completely as possible.

3. Recruitment of subjects

All subjects recruited for this study had been on one of the previously listed ongoing clinical trials. All participants were considered without consideration of race, sex, ethnicity, sexual orientation, or HIV risk factor. Women and members of minority groups were actively recruited to ensure representation and reflect to the best of our ability disease patterns in the local population. Patients enrolled voluntarily in this study. Decisions to either participate or not did not effect that individual's status in the ongoing studies.

4. Subject discontinuation

Subjects, if any, experiencing a Grade 3 or 4 toxicity that could not be excluded as being due to the vaccine(s) exposure were removed from study. Subjects could withdraw at any time. This decision did not effect the ability to receive further care at the Rockefeller University Hospital.

O. Virology

Plasma HIV-1 RNA levels were monitored with the Ultrasensitive RT PCR Assay (Roche) and the Bayer signal amplification assay (version 3.0) as per manufacturer's instructions.

Other details of monitoring are described above.

P. Therapy discontinuation post vaccine

Of the 6 subjects completing the 180 days protocol, 4 elected to discontinue antiretroviral therapy 1 week after the last vaccine at which time HIV-1 plasma RNA levels were measured. Subjects who discontinued therapy include 1306, 1308, 1309, and 1310 and 3002. Of note, one subject had a 5^{th} vaccine injection on day 210 and discontinued therapy one week later.

Baseline characteristics at the initiation of anti-retroviral therapy are shown in Table 4:

Table 4

Subject	Days to treatment	Log HIV-1 RNA	CD cell count	CD4/CD8 ratio
1309	90	4.2	500	0.97
1306	30	5.33	546	0.24
1308	7	6.2	432	0.92
1310	100	3.99	532	0.87

Subjects 1308 and 3002 did not respond to vaccination with an increase in the level of CD8+ IFNγ secreting cells to HIV specific antigens presented in the context of vaccinia. Subject 1310 did respond with an increase in levels of CD8+ IFNγ-secreting cells specific for Gag. Subjects 1306 and 1309 responded with an increase in CD8+ IFN-γ-secreting cells specific for more than 1 HIV-1 specific antigen (see Figure 4). Day 0 refers to the day that subjects discontinued therapy. Period of vaccination occurred during days –217 to 0. Post-discontinuation levels of CTLe are similarly displayed.

Post-therapy discontinuation subjects 1310 and 1306 rebounded after 68 and 85 days respectively. The subjects 1308 and 1310 rebounded within 23 and 13 days of therapy cessation. Furthermore the initial doubling times (t_2) of plasma viremia post therapy cessation were 4.5 and 3.2 days respectively, whereas the subjects who rebounded rapidly had a t_2 of approximately 1.5 days. The virology data for the 4 subjects are shown in Figure 5. It is clear that Subjects 1309 and 1306 not only exhibit a delayed rebound but the mean HIV-1 RNA levels post rebound are also significantly lower than in rapidly rebounding individuals.

Post discontinuation virology data is shown in Table 6:

Table 6

Subject	Days to detectable HIV-1 RNA	Log peak viremia	Time to peak viremia	Current log HIV-1 RNA	Days off therapy
1310	68	2.93	95	2.91	239
1306	85	3.73	145	3.73	145
1308	23	4.15	77	3.55	132
1310	13	4.95	105	4.77	135

Safety of vCP1452•Grade 3 or 4 toxicities 0/8

•Significant systemic toxicities	0/8
•Local tenderness	8/8
•Swelling, redness or induration at the site of vaccination	0/8
•Evidence of activation of virus replication	0/8
•Worsening of baseline adverse events associated with chronic antiretroviral therapy	0/8

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We Claim:

1. A method of permitting cessation of antiviral therapy on HIV-infected patients undergoing antiviral therapy and having a controlled level of viremia without virus rebound, with delayed rebound, and/or with a decrease in the post rebound set point, the method comprising administering to the subject one or a plurality of nucleic acid-based vaccines that enter the patient's cells and intracellularly produce one or a plurality of HIV-specific immunogens for presentation on the cell's MHC class I and MHC class II molecules in an amount sufficient to stimulate an HIV-specific CD8+ and CD4+ responses.

2. The method according to claim 1 wherein the patient has a viral load of less than 10,000 viral copies per ml of plasma and a CD4+ T-cell count of above 300 cells/ml before administration of vaccine.

3. The method according to claim 1 wherein the patient has a viral load of less than 5,000 viral copies per ml of plasma CD4+ T-cell count of above 300 cells/ml before administration of vaccine.

4. The method according to claim 1 wherein the patient has a viral load of less than 1,000 viral copies per ml of plasma CD4+ T-cell count of above 300 cells/ml before administration of vaccine.

5. The method according to claim 1 wherein the patient has a viral load of less than 10,000 viral copies per ml of plasma CD4+ T-cell count of above 500 cells/ml before administration of vaccine.

6. The method according to claim 1 wherein the patient has a viral load of less than 5,000 viral copies per ml of plasma CD4+ T-cell count of above 500 cells/ml before administration of vaccine.

7. The method according to claim 1 wherein the patient has a viral load of less than 1,000 viral copies per ml of plasma CD4+ T-cell count of above 500 cells/ml before administration of vaccine.

8. The method according to claim 1 wherein the patient exhibits CD4+ and/or CD8+ T-cell responses to HIV.

9. The method according to claim 1 wherein the patient exhibits CD4+ and CD8+ T-cell responses to envelope epitopes.

10. The method according to claim 1 wherein the patient exhibits CD4+ and CD8+ T cell responses to Gag epitopes.

11. The method according to claim 1 wherein the patient has lost his CD4+ and/or CD8+ T cell responses to HIV antigens.

12. The method according to claim 1 wherein the patient has lost his CD4+ and CD8+ T cell responses to envelope and Gag HIV epitopes.

13. The method according to claim 1 wherein the HIV specific immunogen is gp120.

14. The method according to claim 1 wherein the HIV-specific immunogen is Gag.

15. The method according to claim 1 wherein the nucleic acid-based vaccine comprises one or a plurality of naked DNAs encoding one or a plurality of HIV-specific immunogens.

16. The method according to claim 1 wherein the nucleic acid-based vaccine comprises one or a plurality of DNA vectors encoding one or a plurality of HIV-specific immunogens.

17. The method according to claim 16 wherein the DNA vector is a recombinant virus.

18. The method according to claim 16 wherein the DNA vector is a recombinant attenuated virus.

19. The method according to claim 17 wherein the recombinant attenuated virus is selected from the group consisting of adenoviruses, adeno-associated viruses, human influenza viruses, herpes simplex virus (HSV), coksackie viruses, vesicular stomatitis viruses (VSV), and alphaviruses.

20. The method according to claim 17 wherein the recombinant attenuated virus is a poxvirus.

21. The method according to claim 20 wherein the recombinant attenuated virus is selected from the group consisting of vaccinia, avipox, fowlpox, and canarypox.

22. The method according to claim 21 wherein the recombinant attenuated virus is NYVAC or ALVAC.

23. The method according to claim 17 wherein the recombinant attenuated virus is MVA.

24. The method according to claim 17 wherein the HIV-specific immunogen is a structural protein.

25. The method according to claim 24 wherein the HIV-specific immunogen is a structural protein selected from the group consisting of gp 160, gp 120, gp 41, and Gag.

26. The method according to claim 17 wherein the HIV-specific immunogen is a non-structural protein.

27. The method according to claim 26 wherein the HIV-specific immunogen is a non-structural protein encoded by a gene selected from the group consisting of *rev, tat, nef, vif,* and *vpr*.

28. The method according to claim 17 wherein the HIV-specific immunogen is selected from the group consisting of HIV-I Gag, gp120, NefCTL, PolCTL epitopes.

29. The method according to claim 17 wherein the HIV-specific immunogen presents at least one epitope selected from the group consisting of ELDKWA, LDKW, Nef1, Nef2, the V3 loop, Pol1, Pol2 and Pol3.

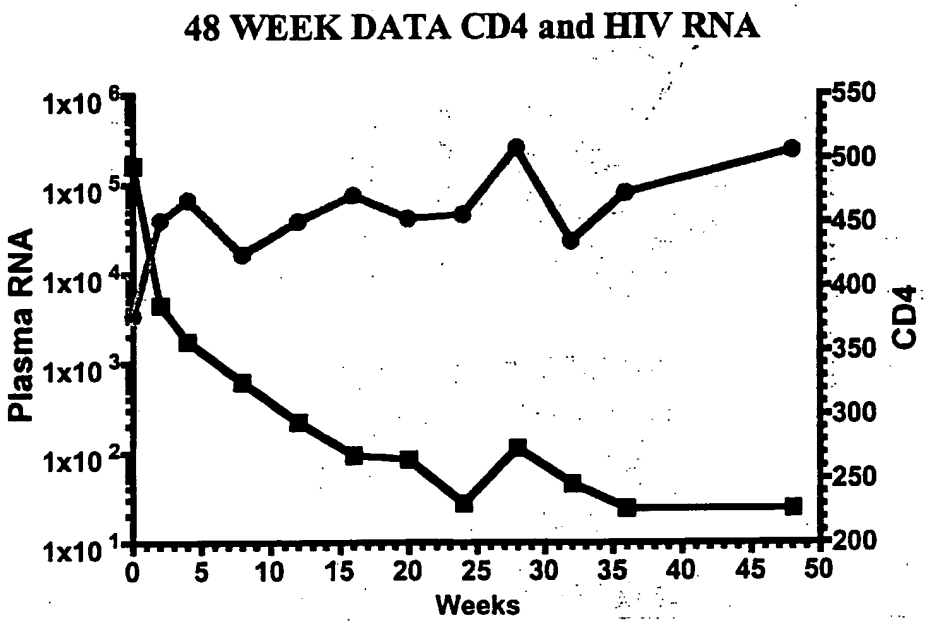
30. The method according to claim 17 wherein the HIV-specific immunogen presents at least one epitope of a peptide selected from the group consisting of gp 160, gp 120, gp 41, Gag, and at least one protein encoded by the *rev, tat, nef, vif,* or *vpr* gene.

31. The method according to claim 1 wherein the nucleic acid-based vaccine comprises a construct selected from the group consisting of vCP1452, vCP1433, vCP125, vCP205, and vCP300.

32. The method according to any one of claims 17, 20, 22, 25, 27, and 30 wherein the vaccine is administered, simultaneously or sequentially, with a soluble HIV antigen.

33. The method according to claim 32, wherein the soluble HIV antigen is gp160.

34. The method according to claim 32, wherein the soluble HIV antigen is recombinant gp160MN/LAI.



(Plasma HIV RNA □ CD 4 cell count O)

Fig. 1

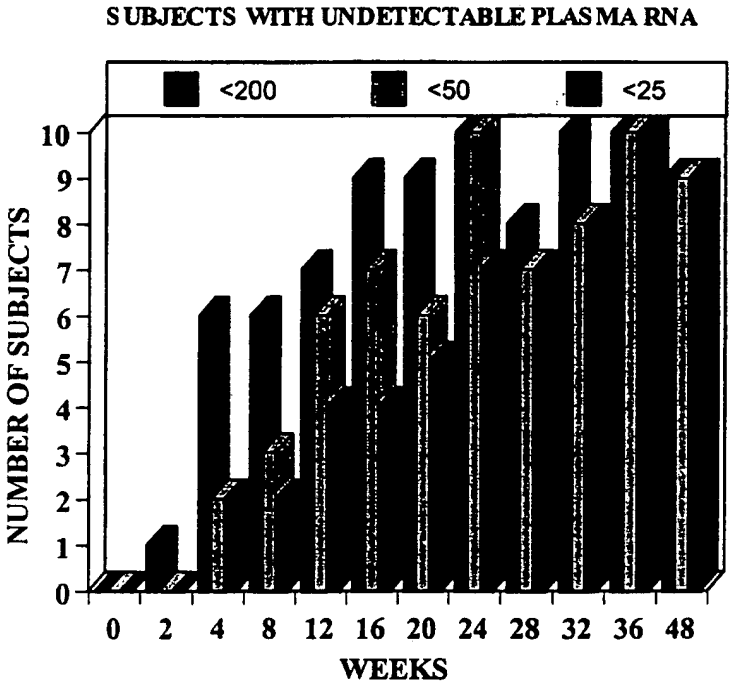

SUBJECTS WITH UNDETECTABLE PLASMA RNA
<200
<50
<25
NUMBER OF SUBJECTS
0
1
2
3
4
5
6
7
8
9
10
0 2 4 8 12 16 20 24 28 32 36 48
WEEKS

Fig. 2

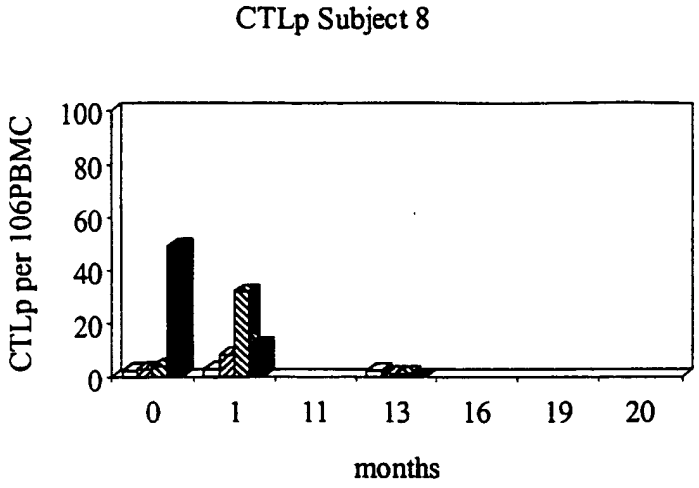

CTLp Subject 8
CTLp per 106PBMC
100
80
60
40
20
0
0
1
11
13
16
19
20
months

Fig. 3A

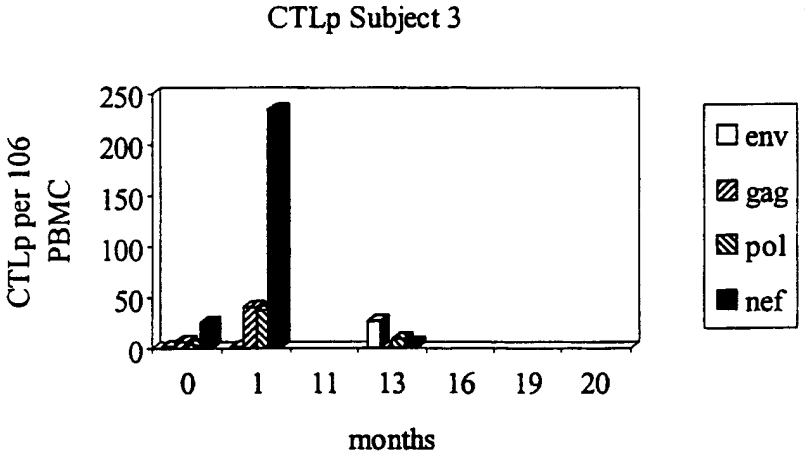
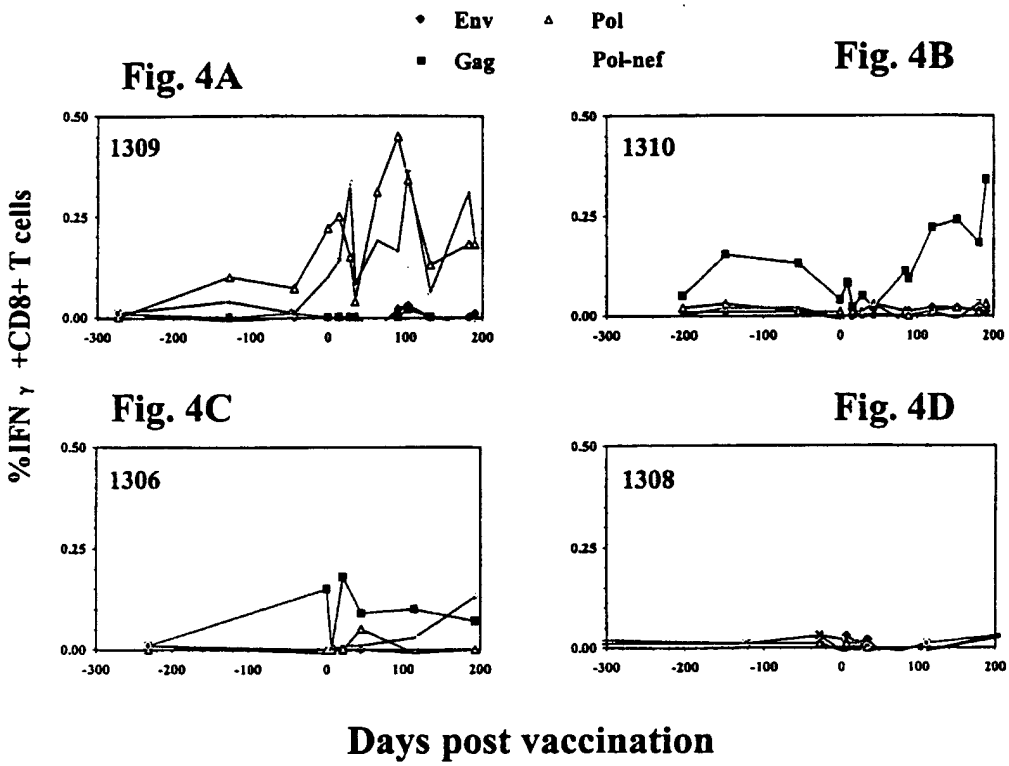
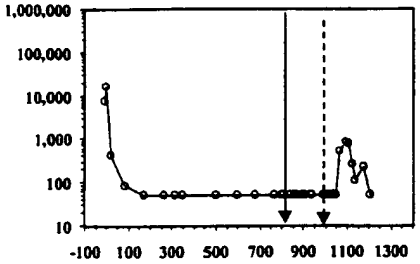

CTLp Subject 3
CTLp per 106 PBMC
250
200
150
100
50
0
0
1
11
13
16
19
20
months
env
gag
pol
nef

Fig. 3B


Env
Pol
Gag
Pol-nef
Fig. 4A
Fig. 4B
Fig. 4C
Fig. 4D
1309
1310
1306
1308
%IFN γ +CD8+ T cells
0.50
0.25
0.00
-300
-200
-100
0
100
200
Days post vaccination

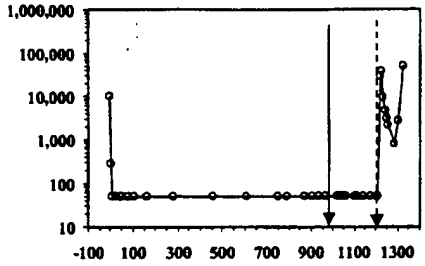
1309

Fig. 5A



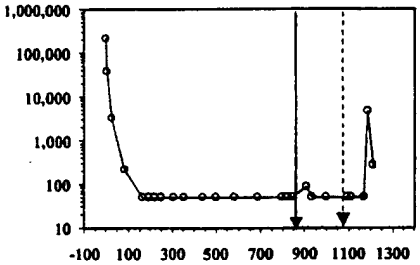
1310

Fig. 5B



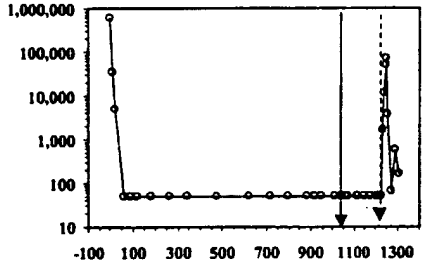
1306

Fig. 5C



1308

Fig. 5D



Plasma viremia

Study Days

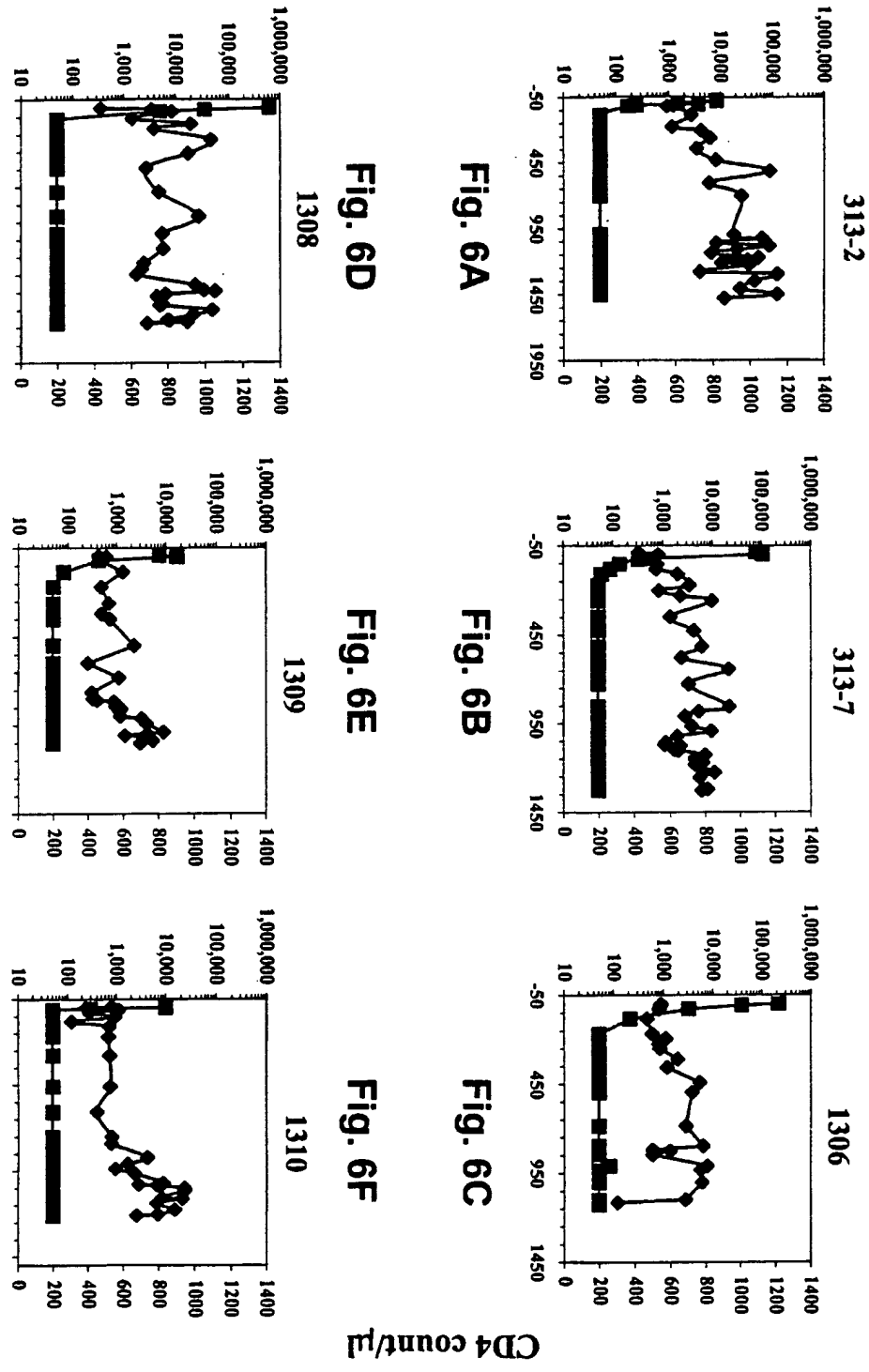


Fig. 6A Fig. 6B Fig. 6C

Fig. 6D Fig. 6E Fig. 6F

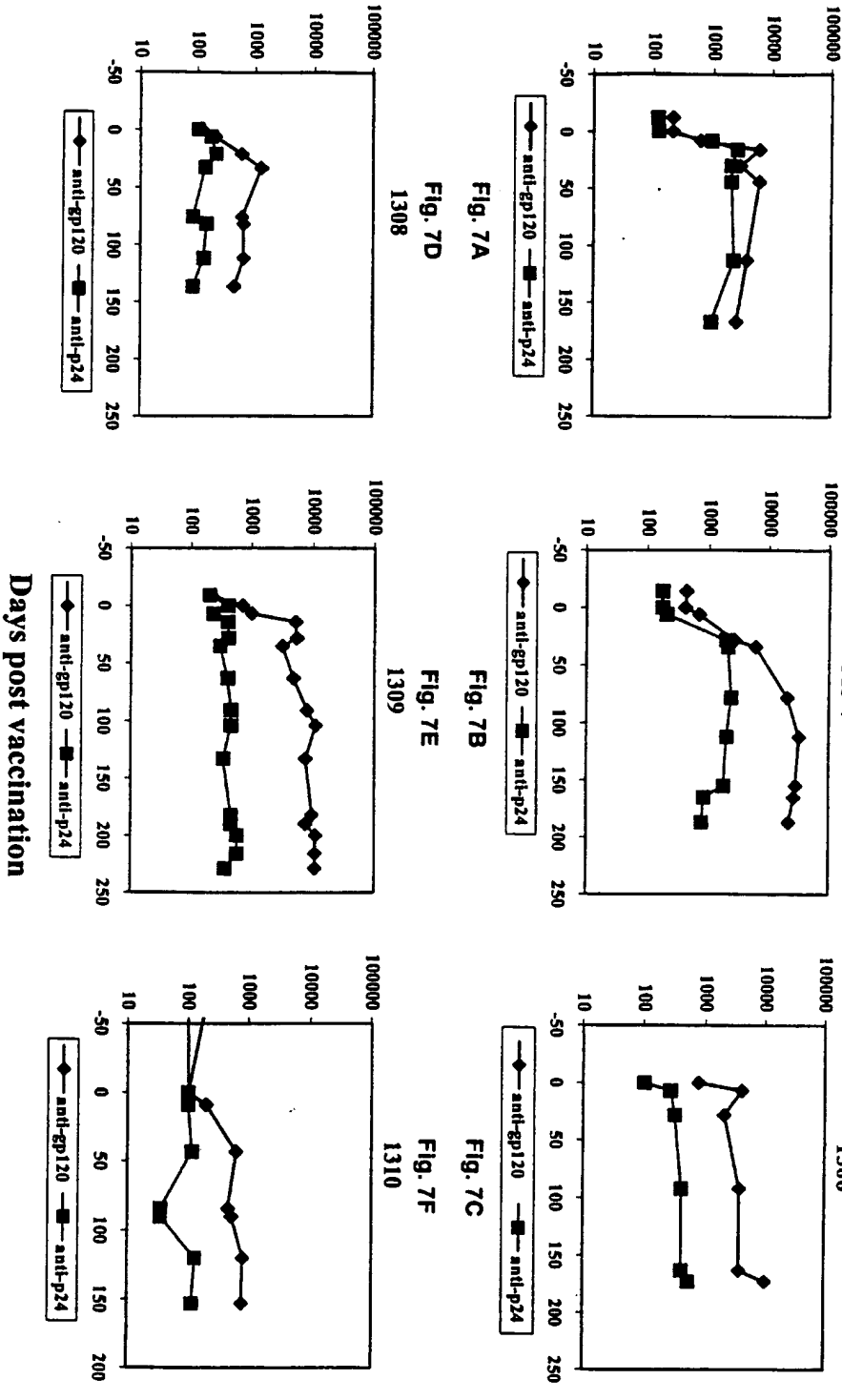


Fig. 7A

Fig. 7B

Fig. 7C

Fig. 7D

Fig. 7E

Fig. 7F

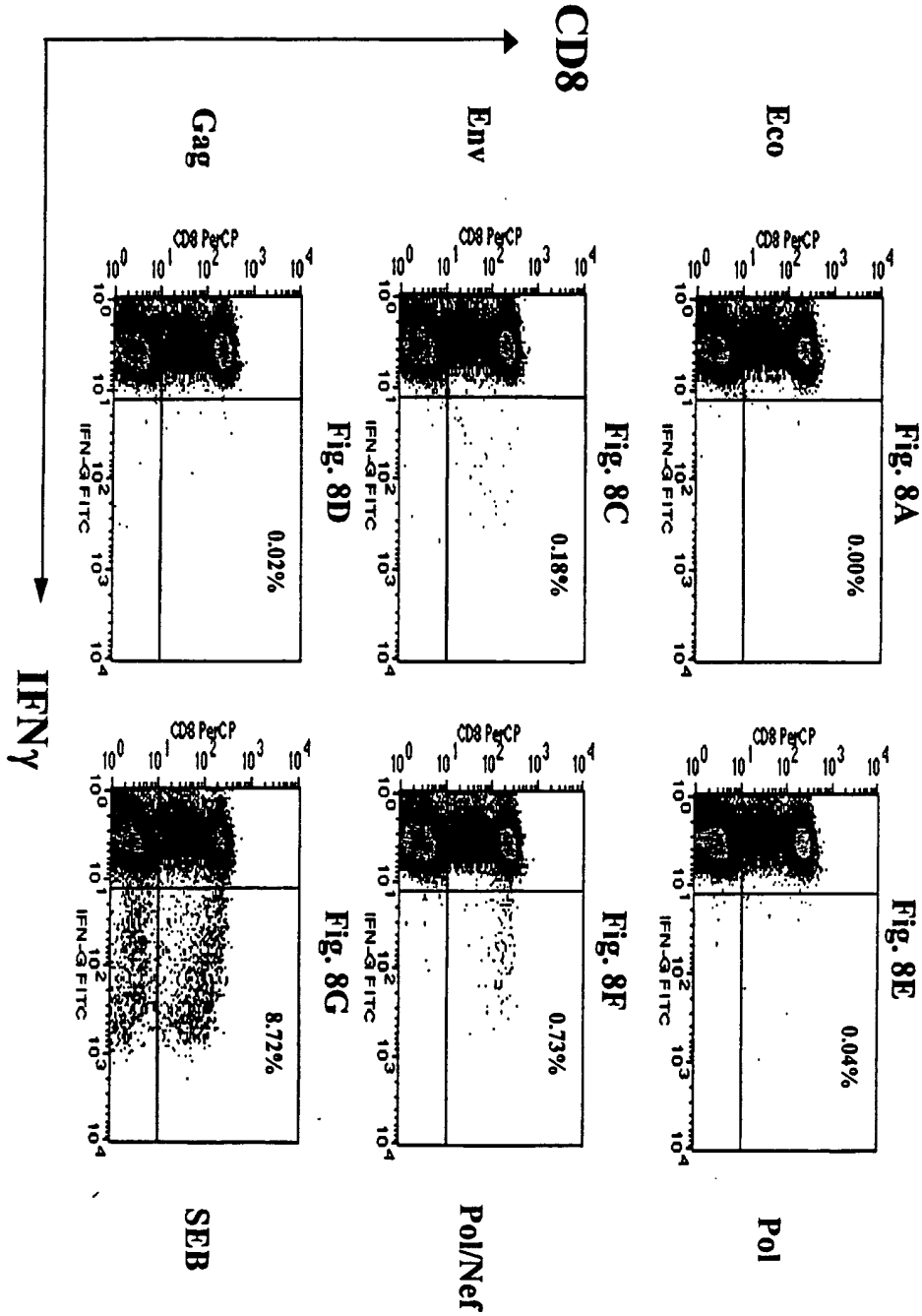


Fig. 8A

Fig. 8C

Fig. 8D

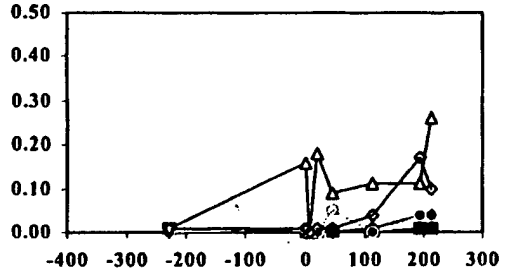
Fig. 8E

Fig. 8F

Fig. 8G

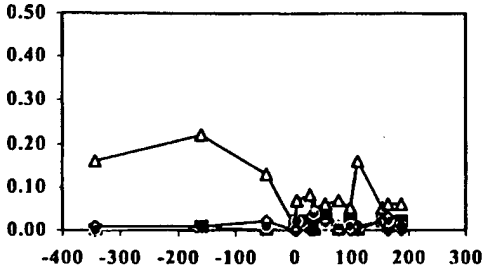
1306

Fig. 9A



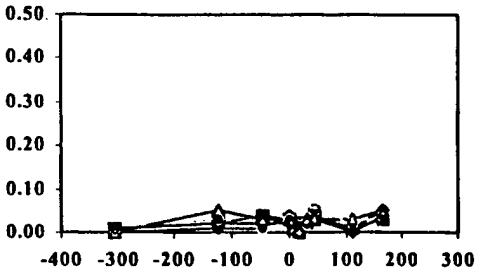
313-7

Fig. 9B



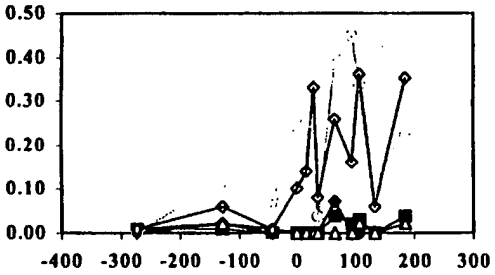
313-2

Fig. 9C



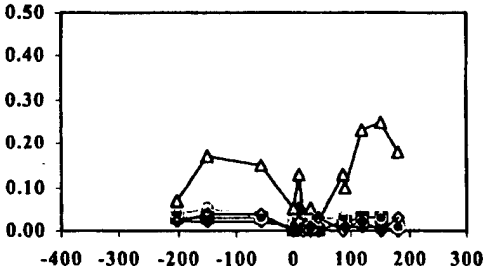
1309

Fig. 9D



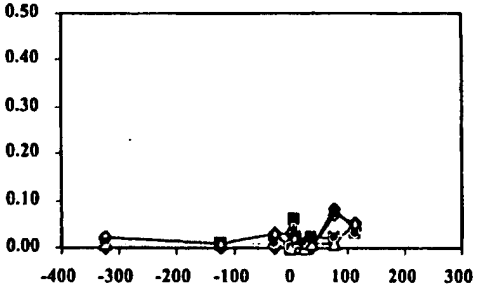
1310

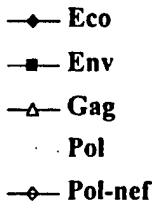
Fig. 9E



1308

Fig. 9F





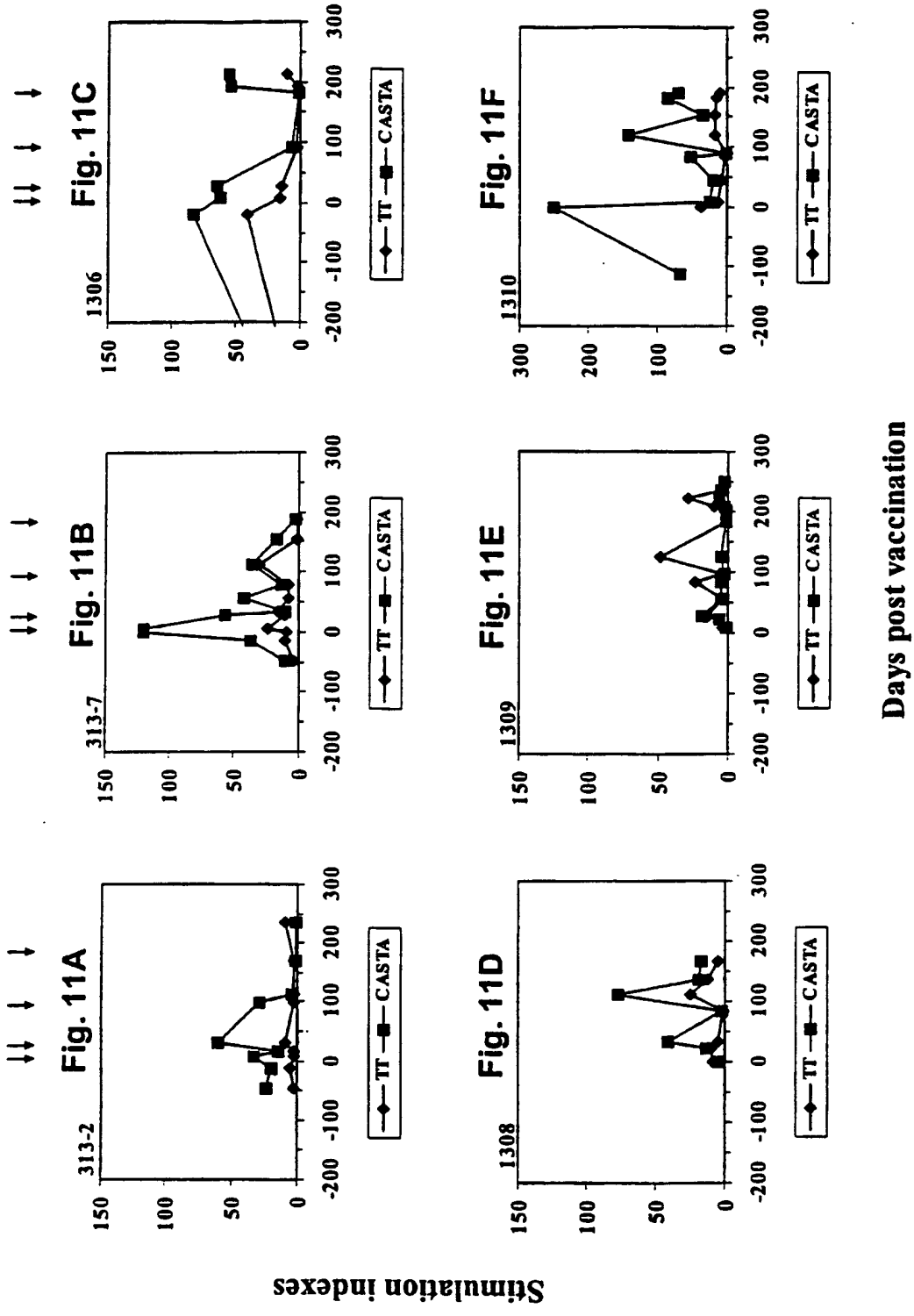


Fig. 11A
313-2
Fig. 11B
313-7
Fig. 11C
1306
Fig. 11D
1308
Fig. 11E
1309
Fig. 11F
1310
TT
CASTA
150
100
50
0
-200
-100
0
100
200
300
Stimulation indexes
Days post vaccination

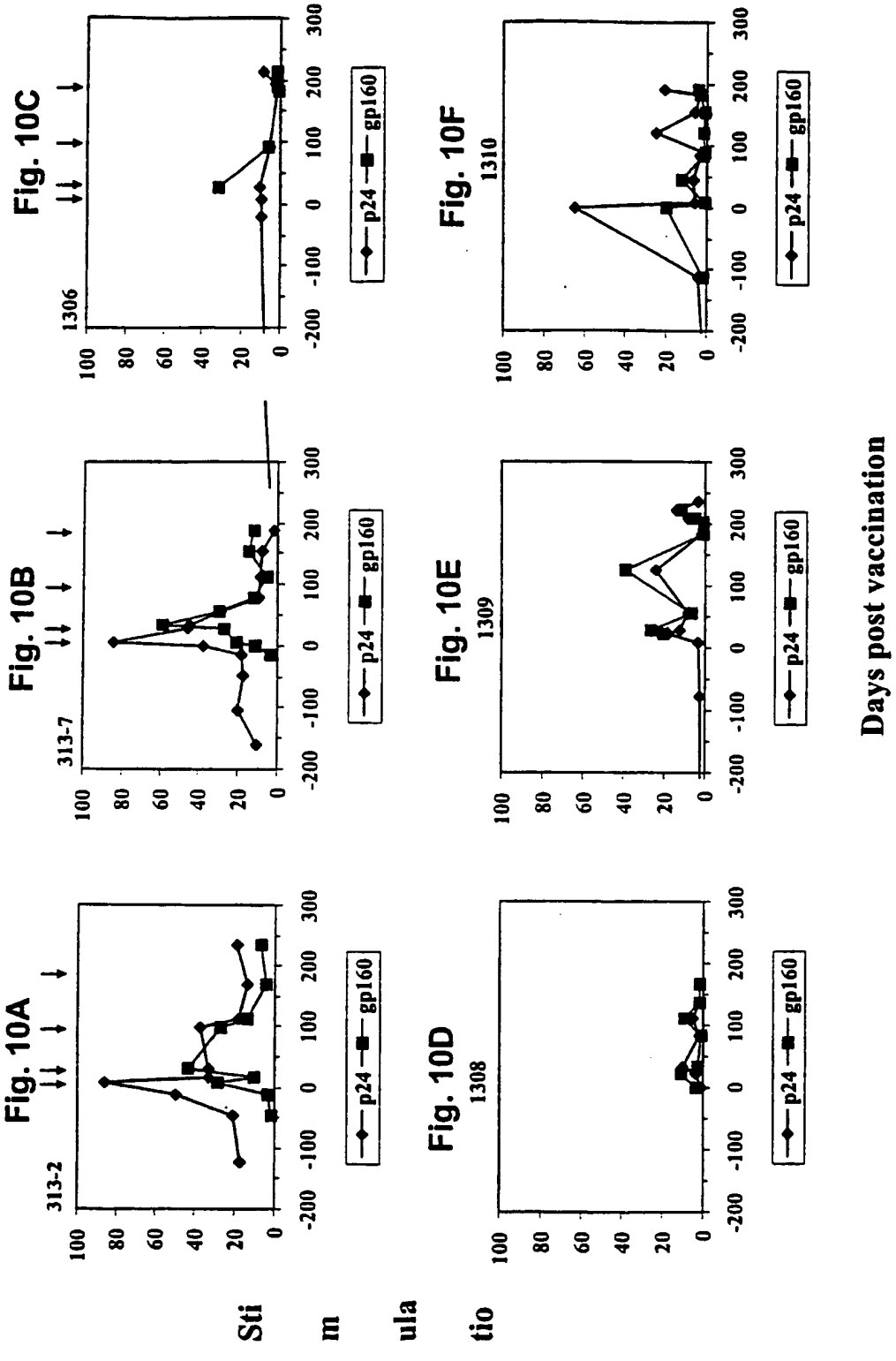


Fig. 10A
313-2
Fig. 10B
313-7
Fig. 10C
1306
Fig. 10D
1308
Fig. 10E
1309
Fig. 10F
1310
p24
gp160
Stimulation
Days post vaccination

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/02766

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/70 A61K39/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 01 08702 A (FRANCHINI GENOVEFFA ;NACSA JANOS (US); US HEALTH (US); HEL ZDENEK) 8 February 2001 (2001-02-08) the whole document	1-34
X	WO 98 08539 A (CHIRON CORP) 5 March 1998 (1998-03-05) the whole document	1-34
X	B. ROSENWIRTH ET AL., : "An anti-HIV strategy combining chemotherapy and therapeutic vaccination" J MED PRIMATOL, vol. 28, no. 4-5, 1999, pages 195-205, XP000982240	1
Y	the whole document	2-34
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
22 May 2001	08/06/2001

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

▶ national Application No
PCT/US 01/02766

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GOTCH F ET AL: "THERAPEUTIC VACCINES IN HIV.1 INFECTION" IMMUNOLOGICAL REVIEWS,MUNKSGAARD,XX, vol. 170, 1999, pages 173-182, XP000982295 ISSN: 0105-2896	1
Y	the whole document	2-34
X	M. JOHN ET AL., : "Control of HIV replication by cytotoxic T-lymphocyte responses" JOURNAL OF HIV THERAPY, vol. 4, no. 4, 1999, pages 91-97, XP000982254	1
Y	the whole document, in particular paragraph bridging pages 95-96	2-34
X	MACGREGOR R R ET AL: "FIRST HUMAN TRIAL OF A DNA-BASED VACCINE FOR TREATMENT OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTION: SAFETY AND HOST RESPONSE" JOURNAL OF INFECTIOUS DISEASES,CHICAGO, IL,US, vol. 178, no. 1, July 1998 (1998-07), pages 92-100, XP000982247 ISSN: 0022-1899	1
Y	last paragraph on page 99	2-34
X	R. HOFF AND J. MCNAMARA: "Therapeutic vaccines for preventing AIDS : their use with HAART" THE LANCET, vol. 353, 22 May 1999 (1999-05-22), pages 1723-1724, XP002168002 the whole document	1
A	PIALOUX ET AL: "A Prime-Boost Approach to HIV Preventive Vaccine Using a Recombinant Canarypox Virus Expressing Glycoprotein 160 (MN) followed by a Recombinant Glycoprotein 160 (MN/LAI)" AIDS RESEARCH AND HUMAN RETROVIRUSES,US,MARY ANN LIEBERT, vol. 11, no. 3, 1995, pages 373-381, XP002079474 ISSN: 0889-2229 the whole document	1-34

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 01/02766

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0108702 A	08-02-2001	NONE	
WO 9808539 A	05-03-1998	AU 4088697 A	19-03-1998
		EP 0942747 A	22-09-1999

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(74) Agent: **PRIVETT, Kathryn, Louise**; Corporate Intellectual Property, SmithKline Beecham, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).

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(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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WO 01/54719 A2

(54) Title: NOVEL USE

(57) Abstract: The invention provides the use of a) an HIV Tat protein or polynucleotide; or b) an HIV Nef protein or polynucleotide; or c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or polynucleotide (Nef-Tat); and an HIV gp120 protein or polynucleotide in the manufacture of a vaccine for the prophylactic or therapeutic immunisation of humans against HIV.

NOVEL USE

DESCRIPTION

The present invention relates to novel uses of HIV proteins in medicine and vaccine compositions containing such HIV proteins. In particular, the invention relates to the use of HIV Tat and HIV gp120 proteins in combination. Furthermore, the invention relates to the use of HIV Nef and HIV gp120 proteins in combination.

HIV-1 is the primary cause of the acquired immune deficiency syndrome (AIDS) which is regarded as one of the world's major health problems. Although extensive research throughout the world has been conducted to produce a vaccine, such efforts thus far have not been successful.

The HIV envelope glycoprotein gp120 is the viral protein that is used for attachment to the host cell. This attachment is mediated by the binding to two surface molecules of helper T cells and macrophages, known as CD4 and one of the two chemokine receptors CCR-4 or CXCR-5. The gp120 protein is first expressed as a larger precursor molecule (gp160), which is then cleaved post-translationally to yield gp120 and gp41. The gp120 protein is retained on the surface of the virion by linkage to the gp41 molecule, which is inserted into the viral membrane.

The gp120 protein is the principal target of neutralizing antibodies, but unfortunately the most immunogenic regions of the proteins (V3 loop) are also the most variable parts of the protein. Therefore, the use of gp120 (or its precursor gp160) as a vaccine antigen to elicit neutralizing antibodies is thought to be of limited use for a broadly protective vaccine. The gp120 protein does also contain epitopes that are recognized by cytotoxic T lymphocytes (CTL). These effector cells are able to eliminate virus-infected cells, and therefore constitute a second major antiviral immune mechanism. In contrast to the target regions of neutralizing antibodies some CTL epitopes appear to be relatively conserved among different HIV strains. For this reason gp120 and gp160 are considered to be useful antigenic components in vaccines that aim at eliciting cell-mediated immune responses (particularly CTL).

Non-envelope proteins of HIV-1 have been described and include for example internal structural proteins such as the products of the *gag* and *pol* genes and, other non-structural proteins such as Rev, Nef, Vif and Tat (Greene et al., New England J. Med, 324, 5, 308 et seq (1991) and Bryant et al. (Ed. Pizzo), Pediatr. Infect. Dis. J., 11, 5, 390 et seq (1992).

HIV Tat and Nef proteins are early proteins, that is, they are expressed early in infection and in the absence of structural protein.

In a conference presentation (C. David Pauza, Immunization with Tat toxoid attenuates SHIV89.6PD infection in rhesus macaques, 12th Cent Gardes meeting, Marnes-La-Coquette, 26.10.1999), experiments were described in which rhesus macaques were immunised with Tat toxoid alone or in combination with an envelope glycoprotein gp160 vaccine combination (one dose recombinant vaccinia virus and one dose recombinant protein). However, the results observed showed that the presence of the envelope glycoprotein gave no advantage over experiments performed with Tat alone.

However, we have found that a Tat- and/or Nef-containing immunogen (especially a Nef-Tat fusion protein) acts synergistically with gp120 in protecting rhesus monkeys from a pathogenic challenge with chimeric human-simian immunodeficiency virus (SHIV). To date the SHIV infection of rhesus macaques is considered to be the most relevant animal model for human AIDS. Therefore, we have used this preclinical model to evaluate the protective efficacy of vaccines containing a gp120 antigen and a Nef- and Tat-containing antigen either alone or in combination. Analysis of two markers of viral infection and pathogenicity, the percentage of CD4-positive cells in the peripheral blood and the concentration of free SHIV RNA genomes in the plasma of the monkeys, indicated that the two antigens acted in synergy. Immunization with either gp120 or NefTat + SIV Nef alone did not result in any difference compared to immunization with an adjuvant alone. In contrast, the administration of the combination of gp120 and NefTat + SIV Nef, antigens resulted in a marked improvement of the two above-mentioned parameters in all animals of those particular experimental group.

Thus, according to the present invention there is provided a new use of HIV Tat and/or Nef protein together with HIV gp120 in the manufacture of a vaccine for the prophylactic or therapeutic immunisation of humans against HIV.

As described above, the NefTat protein, the SIV Nef protein and gp120 protein together give an enhanced response over that which is observed when either NefTat + SIV Nef, or gp120 are used alone. This enhanced response, or synergy can be seen in a decrease in viral load as a result of vaccination with these combined proteins. Alternatively, or additionally the enhanced response manifests itself by a maintenance of CD4+ levels over those levels found in the absence of vaccination with HIV NefTat, SIV Nef and HIV gp120. The synergistic effect is attributed to the combination of gp120 and Tat, or gp120 and Nef, or gp120 and both Nef and Tat.

The addition of other HIV proteins may further enhance the synergistic effect, which was observed between gp120 and Tat and/or Nef. These other proteins may also act synergistically with individual components of the gp120, Tat and/or Nef-containing vaccine, not requiring the presence of the full original antigen combination. The additional proteins may be regulatory proteins of HIV such as Rev, Vif, Vpu, and Vpr. They may also be structural proteins derived from the HIV *gag* or *pol* genes.

The HIV *gag* gene encodes a precursor protein p55, which can assemble spontaneously into immature virus-like particles (VLPs). The precursor is then proteolytically cleaved into the major structural proteins p24 (capsid) and p18 (matrix), and into several smaller proteins. Both the precursor protein p55 and its major derivatives p24 and p18 may be considered as appropriate vaccine antigens which may further enhance the synergistic effect observed between gp120 and Tat and/or Nef. The precursor p55 and the capsid protein p24 may be used as VLPs or as monomeric proteins.

The HIV Tat protein in the vaccine of the present invention may, optionally be linked to an HIV Nef protein, for example as a fusion protein.

The HIV Tat protein, the HIV Nef protein or the NefTat fusion protein in the present invention may have a C termir al Histidine tail which preferably comprises between 5-10 Histidine residues. The presence of an histidine (or 'His') tail aids purification.

In a preferred embodiment the proteins are expressed with a Histidine tail comprising between 5 to 10 and preferably six Histidine residues. These are advantageous in aiding purification. Separate expression, in yeast (Saccharomyces cerevisiae), of Nef (Macreadie I.G. et al., 1993, Yeast 9 (6) 565-573) and Tat (Braddock M et al., 1989, Cell 58 (2) 269-79) has been reported. Nef protein and the Gag proteins p55 and p18 are myristilated. The expression of Nef and Tat separately in a Pichia expression system (Nef-His and Tat-His constructs), and the expression of a fusion construct Nef-Tat-His have been described previously in WO99/16884.

The DNA and amino acid sequences of representative Nef-His (Seq. ID. No.s 8 and 9), Tat-His (Seq. ID. No.s 10 and 11)and of Nef-Tat-His fusion proteins (Seq. ID. No.s 12 and 13) are set forth in Figure 1.

The HIV proteins of the present invention may be used in their native conformation, or more preferably, may be modified for vaccine use. These modifications may either be required for technical reasons relating to the method of purification, or they may be used to biologically inactivate one or several functional properties of the Tat or Nef protein. Thus the invention encompasses derivatives of HIV proteins which may be, for example mutated proteins. The term 'mutated' is used herein to mean a molecule which has undergone deletion, addition or substitution of one or more amino acids using well known techniques for site directed mutagenesis or any other conventional method.

For example, a mutant Tat protein may be mutated so that it is biologically inactive whilst still maintaining its immunogenic epitopes. One possible mutated tat gene, constructed by D.Clements (Tulane University), (originating from BH10 molecular clone) bears mutations in the active site region (Lys41→Ala)and in RGD motif (Arg78→Lys and Asp80→Glu) (Virology 235: 48-64, 1997).

A mutated Tat is illustrated in Figure 1 (Seq. ID. No.s 22 and 23) as is a Nef-Tat Mutant-His (Seq. ID. No.s 24 and 25).

The HIV Tat or Nef proteins in the vaccine of the present invention may be modified by chemical methods during the purification process to render the proteins stable and monomeric. One method to prevent oxidative aggregation of a protein such as Tat or Nef is the use of chemical modifications of the protein's thiol groups. In a first step the disulphide bridges are reduced by treatment with a reducing agent such as DTT, beta-mercaptoethanol, or gluthatione. In a second step the resulting thiols are blocked by reaction with an alkylating agent (for example, the protein can be carboxyamidated/carbamidomethylated using iodoacetamide). Such chemical modification does not modify functional properties of Tat or Nef as assessed by cell binding assays and inhibition of lymphoproliferation of human peripheral blood mononuclear cells.

The HIV Tat protein and HIV gp120 proteins can be purified by the methods outlined in the attached examples.

The vaccine of the present invention will contain an immunoprotective or immunotherapeutic quantity of the Tat and/or Nef or NefTat and gp120 antigens and may be prepared by conventional techniques.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

The amount of protein in the vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed. Generally, it is expected that each dose will comprise 1-1000 μg of each

protein, preferably 2-200 μg, most preferably 4-40 μg of Tat or Nef or NefTat and preferably 1-150 μg, most preferably 2-25 μg of gp120. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. One particular example of a vaccine dose will comprise 20 μg of NefTat and 5 or 20 μg of gp120. Following an initial vaccination, subjects may receive a boost in about 4 weeks, and a subsequent second booster immunisation.

The proteins of the present invention are preferably adjuvanted in the vaccine formulation of the invention. Adjuvants are described in general in Vaccine Design – the Subunit and Adjuvant Approach, edited by Powell and Newman, Plenum Press, New York, 1995.

Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

In the formulation of the invention it is preferred that the adjuvant composition induces a preferential Th1 response. However it will be understood that other responses, including other humoral responses, are not excluded.

An immune response is generated to an antigen through the interaction of the antigen with the cells of the immune system. The resultant immune response may be broadly distinguished into two extreme catagories, being humoral or cell mediated immune responses (traditionally characterised by antibody and cellular effector mechanisms of protection respectively). These categories of response have been termed Th1-type responses (cell-mediated response), and Th2-type immune responses (humoral response).

Extreme Th1-type immune responses may be characterised by the generation of antigen specific, haplotype restricted cytotoxic T lymphocytes, and natural killer cell responses. In mice Th1-type responses are often characterised by the generation of

antibodies of the IgG2a subtype, whilst in the human these correspond to IgG1 type antibodies. Th2-type immune responses are characterised by the generation of a broad range of immunoglobulin isotypes including in mice IgG1, IgA, and IgM.

It can be considered that the driving force behind the development of these two types of immune responses are cytokines, a number of identified protein messengers which serve to help the cells of the immune system and steer the eventual immune response to either a Th1 or Th2 response. Thus high levels of Th1-type cytokines tend to favour the induction of cell mediated immune responses to the given antigen, whilst high levels of Th2-type cytokines tend to favour the induction of humoral immune responses to the antigen.

It is important to remember that the distinction of Th1 and Th2-type immune responses is not absolute. In reality an individual will support an immune response which is described as being predominantly Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 +ve T cell clones by Mosmann and Coffman (*Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, 7, p145-173*). Traditionally, Th1-type responses are associated with the production of the INF-γ and IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of Th1-type immune responses are not produced by T-cells, such as IL-12. In contrast, Th2- type responses are associated with the secretion of IL-4, IL-5, IL-6, IL-10 and tumour necrosis factor-β(TNF-β).

It is known that certain vaccine adjuvants are particularly suited to the stimulation of either Th1 or Th2 - type cytokine responses. Traditionally the best indicators of the Th1:Th2 balance of the immune response after a vaccination or infection includes direct measurement of the production of Th1 or Th2 cytokines by T lymphocytes *in vitro* after restimulation with antigen, and/or the measurement of the IgG1:IgG2a ratio of antigen specific antibody responses.

Thus, a Th1-type adjuvant is one which stimulates isolated T-cell populations to produce high levels of Th1-type cytokines when re-stimulated with antigen *in vitro*, and induces antigen specific immunoglobulin responses associated with Th1-type isotype.

Preferred Th1-type immunostimulants which may be formulated to produce adjuvants suitable for use in the present invention include and are not restricted to the following.

Monophosphoryl lipid A, in particular 3-de-O-acylated monophosphoryl lipid A (3D-MPL), is a preferred Th1-type immunostimulant for use in the invention. 3D-MPL is a well known adjuvant manufactured by Ribi Immunochem, Montana. Chemically it is often supplied as a mixture of 3-de-O-acylated monophosphoryl lipid A with either 4, 5, or 6 acylated chains. It can be purified and prepared by the methods taught in GB 2122204B, which reference also discloses the preparation of diphosphoryl lipid A, and 3-O-deacylated variants thereof. Other purified and synthetic lipopolysaccharides have been described (US 6,005,099 and EP 0 729 473 B1; Hilgers *et al.*, 1986, *Int.Arch.Allergy.Immunol.*, 79(4):392-6; Hilgers *et al.*, 1987, Immunology, 60(1):141-6; and EP 0 549 074 B1). A preferred form of 3D-MPL is in the form of a particulate formulation having a small particle size less than 0.2μm in diameter, and its method of manufacture is disclosed in EP 0 689 454.

Saponins are also preferred Th1 immunostimulants in accordance with the invention. Saponins are well known adjuvants and are taught in: Lacaille-Dubois, M and Wagner H. (1996. A review of the biological and pharmacological activities of saponins. Phytomedicine vol 2 pp 363-386). For example, Quil A (derived from the bark of the South American tree Quillaja Saponaria Molina), and fractions thereof, are described in US 5,057,540 and "Saponins as vaccine adjuvants", Kensil, C. R., *Crit Rev Ther Drug Carrier Syst*, 1996, 12 (1-2):1-55; and EP 0 362 279 B1. The haemolytic saponins QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants, and the method of their production is disclosed in US Patent No. 5,057,540 and EP 0 362 279 B1. Also described in these references is the use of QS7 (a non-haemolytic fraction of Quil-A) which acts as a potent adjuvant for systemic vaccines. Use of QS21 is further described in Kensil *et al.* (1991. J.

Immunology vol 146, 431-437). Combinations of QS21 and polysorbate or cyclodextrin are also known (WO 99/10008). Particulate adjuvant systems comprising fractions of QuilA, such as QS21 and QS7 are described in WO 96/33739 and WO 96/11711.

Another preferred immunostimulant is an immunostimulatory oligonucleotide containing unmethylated CpG dinucleotides ("CpG"). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. CpG is known in the art as being an adjuvant when administered by both systemic and mucosal routes (WO 96/02555, EP 468520, Davis *et al., J.Immunol*, 1998, 160(2):870-876; McCluskie and Davis, *J.Immunol.*, 1998, 161(9):4463-6). Historically, it was observed that the DNA fraction of BCG could exert an anti-tumour effect. In further studies, synthetic oligonucleotides derived from BCG gene sequences were shown to be capable of inducing immunostimulatory effects (both in vitro and in vivo). The authors of these studies concluded that certain palindromic sequences, including a central CG motif, carried this activity. The central role of the CG motif in immunostimulation was later elucidated in a publication by Krieg, Nature 374, p546 1995. Detailed analysis has shown that the CG motif has to be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA. The immunostimulatory sequence is often: Purine, Purine, C, G, pyrimidine, pyrimidine; wherein the CG motif is not methylated, but other unmethylated CpG sequences are known to be immunostimulatory and may be used in the present invention.

In certain combinations of the six nucleotides a palindromic sequence is present. Several of these motifs, either as repeats of one motif or a combination of different motifs, can be present in the same oligonucleotide. The presence of one or more of these immunostimulatory sequences containing oligonucleotides can activate various immune subsets, including natural killer cells (which produce interferon γ and have cytolytic activity) and macrophages (Wooldrige et al Vol 89 (no. 8), 1977). Other unmethylated CpG containing sequences not having this consensus sequence have also now been shown to be immunomodulatory.

CpG when formulated into vaccines, is generally administered in free solution together with free antigen (WO 96/02555; McCluskie and Davis, *supra*) or covalently conjugated to an antigen (WO 98/16247), or formulated with a carrier such as aluminium hydroxide ((Hepatitis surface antigen) Davis *et al. supra* ; Brazolot-Millan *et al., Proc.Natl.Acad.Sci.*, USA, 1998, 95(26), 15553-8).

Such immunostimulants as described above may be formulated together with carriers, such as for example liposomes, oil in water emulsions, and or metallic salts, including aluminium salts (such as aluminium hydroxide). For example, 3D-MPL may be formulated with aluminium hydroxide (EP 0 689 454) or oil in water emulsions (WO 95/17210); QS21 may be advantageously formulated with cholesterol containing liposomes (WO 96/33739), oil in water emulsions (WO 95/17210) or alum (WO 98/15287); CpG may be formulated with alum (Davis *et al. supra* ; Brazolot-Millan *supra*) or with other cationic carriers.

Combinations of immunostimulants are also preferred, in particular a combination of a monophosphoryl lipid A and a saponin derivative (WO 94/00153; WO 95/17210; WO 96/33739; WO 98/56414; WO 99/12565; WO 99/11241), more particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153. Alternatively, a combination of CpG plus a saponin such as QS21 also forms a potent adjuvant for use in the present invention.

Thus, suitable adjuvant systems include, for example, a combination of monophosphoryl lipid A, preferably 3D-MPL, together with an aluminium salt. An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched in cholesterol containing liposomes (DQ) as disclosed in WO 96/33739.

A particularly potent adjuvant formulation involving QS21, 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210 and is another preferred formulation for use in the invention.

Another preferred formulation comprises a CpG oligonucleotide alone or together with an aluminium salt.

In another aspect of the invention, the vaccine may contain DNA encoding one or more of the Tat, Nef and gp120 polypeptides, such that the polypeptide is generated *in situ*. The DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems such as plasmid DNA, bacteria and viral expression systems. Numerous gene delivery techniques are well known in the art, such as those described by Rolland, Crit. Rev. Therap. Drug Carrier Systems 15:143-198, 1998 and references cited therein. Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter and terminating signal). When the expression system is a recombinant live microorganism, such as a virus or bacterium, the gene of interest can be inserted into the genome of a live recombinant virus or bacterium. Inoculation and *in vivo* infection with this live vector will lead to *in vivo* expression of the antigen and induction of immune responses. Viruses and bacteria used for this purpose are for instance: poxviruses (e.g; vaccinia, fowlpox, canarypox, modified poxviruses e.g. Modified Virus Ankara (MVA)), alphaviruses (Sindbis virus, Semliki Forest Virus, Venezuelian Equine Encephalitis Virus), flaviviruses (yellow fever virus, Dengue virus, Japanese encephalitis virus), adenoviruses, adeno-associated virus, picornaviruses (poliovirus, rhinovirus), herpesviruses (varicella zoster virus, etc), Listeria, Salmonella , Shigella, Neisseria, BCG. These viruses and bacteria can be virulent, or attenuated in various ways in order to obtain live vaccines. Such live vaccines also form part of the invention.

Thus, the Nef, Tat and gp120 components of a preferred vaccine according to the invention may be provided in the form of polynucleotides encoding the desired proteins.

Furthermore, immunisations according to the invention may be performed with a combination of protein and DNA-based formulations. Prime-boost immunisations are considered to be effective in inducing broad immune responses. Adjuvanted protein vaccines induce mainly antibodies and T helper immune responses, while delivery of DNA as a plasmid or a live vector induces strong cytotoxic T lymphocyte (CTL)

responses. Thus, the combination of protein and DNA vaccination will provide for a wide variety of immune responses. This is particularly relevant in the context of HIV, since both neutralising antibodies and CTL are thought to be important for the immune defence against HIV.

In accordance with the invention a schedule for vaccination with gp120, Nef and Tat, alone or in combination, may comprise the sequential ("prime-boost") or simultaneous administration of protein antigens and DNA encoding the above-mentioned proteins. The DNA may be delivered as plasmid DNA or in the form of a recombinant live vector, e.g. a poxvirus vector or any other suitable live vector such as those described herein. Protein antigens may be injected once or several times followed by one or more DNA administrations, or DNA may be used first for one or more administrations followed by one or more protein immunisations.

A particular example of prime-boost immunisation according to the invention involves priming with DNA in the form of a recombinant live vector such as a modified poxvirus vector, for example Modified Virus Ankara (MVA) or a alphavirus, for example Venezuelian Equine Encephalitis Virus followed by boosting with a protein, preferably an adjuvanted protein.

Thus the invention further provides a pharmaceutical kit comprising:

a) a composition comprising one or more of gp120, Nef and Tat proteins together with a pharmaceutically acceptable excipient; and

b) a composition comprising one or more of gp120, Nef and Tat-encoding polynucleotides together with a pharmaceutically acceptable excipient;

with the proviso that at least one of (a) or (b) comprises gp120 with Nef and/or Tat and/or Nef-Tat.

Compositions a) and b) may be administered separately, in any order, or together. Preferably a) comprises all three of gp120, Nef and Tat proteins. Preferably b) comprises all three of gp120, Nef and Tat DNA. Most preferably the Nef and Tat are in the form of a NefTat fusion protein.

In a further aspect of the present invention there is provided a method of manufacture of a vaccine formulation as herein described, wherein the method comprises admixing

a combination of proteins according to the invention. The protein composition may be mixed with a suitable adjuvant and, optionally, a carrier.

Particularly preferred adjuvant and/or carrier combinations for use in the formulations according to the invention are as follows:

i) 3D-MPL + QS21 in DQ

ii) Alum + 3D-MPL

iii) Alum + QS21 in DQ + 3D-MPL

iv) Alum + CpG

v) 3D-MPL + QS21 in DQ + oil in water emulsion

vi) CpG

The invention is illustrated in the accompanying examples and Figures:

EXAMPLES

General

The Nef gene from the Bru/Lai isolate (Cell 40: 9-17, 1985) was selected for the constructs of these experiments since this gene is among those that are most closely related to the consensus Nef .

The starting material for the Bru/Lai Nef gene was a 1170bp DNA fragment cloned on the mammalian expression vector pcDNA3 (pcDNA3/Nef).

The Tat gene originates from the BH10 molecular clone. This gene was received as an HTLV III cDNA clone named pCV1 and described in Science, 229, p69-73, 1985.

The expression of the Nef and Tat genes could be in Pichia or any other host.

Example 1. EXPRESSION OF HIV-1 *nef* AND *tat* SEQUENCES IN PICHIA PASTORIS.

Nef protein, Tat protein and the fusion Nef -Tat were expressed in the methylotrophic yeast *Pichia pastoris* under the control of the inducible alcohol oxidase (AOX1) promoter.

To express these HIV-1 genes a modified version of the integrative vector PHIL-D2 (INVITROGEN) was used. This vector was modified in such a way that expression of heterologous protein starts immediately after the native ATG codon of the AOX1 gene and will produce recombinant protein with a tail of one glycine and six histidines residues . This PHIL-D2-MOD vector was constructed by cloning an oligonucleotide linker between the adjacent AsuII and EcoRI sites of PHIL-D2 vector (see Figure 2). In addition to the His tail, this linker carries NcoI, SpeI and XbaI restriction sites between which *nef*, *tat* and *nef-tat* fusion were inserted.

1.1 CONSTRUCTION OF THE INTEGRATIVE VECTORS pRIT14597 (encoding Nef-His protein), pRIT14598 (encoding Tat-His protein) and pRIT14599 (encoding fusion Nef-Tat-His).

The *nef* gene was amplified by PCR from the pcDNA3/Nef plasmid with primers 01 and 02.

NcoI

PRIMER 01 (Seq ID NO 1): 5'ATCGTCCATG.GGT.GGC.AAG.TGG.T 3'

SpeI

PRIMER 02 (Seq ID NO 2): 5' CGGCTACTAGTGCAGTTCTTGAA 3'

The PCR fragment obtained and the integrative PHIL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose gel and ligated to create the integrative plasmid pRIT14597 (see Figure 2).

The *tat* gene was amplified by PCR from a derivative of the pCV1 plasmid with primers 05 and 04:

SpeI

PRIMER 04 (Seq ID NO 4): 5' CGGCTACTAGTTTCCTTCGGGCCT 3'

NcoI

PRIMER 05 (Seq ID NO 5): 5'ATCGTCCATGGAGCCAGTAGATC 3'

An NcoI restriction site was introduced at the 5' end of the PCR fragment while a SpeI site was introduced at the 3' end with primer 04. The PCR fragment obtained

and the PHIL-D2-MOD vecto· were both restricted by NcoI and SpeI, purified on agarose gel and ligated to create the integrative plasmid pRIT14598.

To construct pRIT14599, a 910bp DNA fragment corresponding to the *nef-tat*-His coding sequence was ligated between the EcoRI blunted(T4 polymerase) and NcoI sites of the PHIL-D2-MOD vector. The *nef-tat*-His coding fragment was obtained by XbaI blunted(T4 polymerase) and NcoI digestions of pRIT14596.

1.2 TRANSFORMATION OF PICHIA PASTORIS STRAIN GS115(his4).

To obtain *Pichia pastoris* strains expressing Nef-His, Tat-His and the fusion Nef-Tat-His, strain GS115 was transformed with linear NotI fragments carrying the respective expression cassettes plus the HIS4 gene to complement his4 in the host genome.Transformation of GS115 with NotI-linear fragments favors recombination at the AOXI locus.

Multicopy integrant clones were selected by quantitative dot blot analysis and the type of integration, insertion (Mut^+phenotype) or transplacement (Mut^sphenotype), was determined.

From each transformation, one transformant showing a high production level for the recombinant protein was selected :

Strain Y1738 (Mut^+ phenotype) producing the recombinant Nef-His protein, a myristylated 215 amino acids protein which is composed of:

- °Myristic acid
- °A methionine, created by the use of NcoI cloning site of PHIL-D2-MOD vector
- °205 a.a. of Nef protein(starting at a.a.2 and extending to a.a.206)
- °A threonine and a serine created by the cloning procedure (cloning at SpeI site of PHIL-D2-MOD vector.
- °One glycine and six histidines.

Strain Y1739 (Mut^+ phenotype) producing the Tat-His protein, a 95 amino acid protein which is composed of:

°A methionine created by the use of NcoI cloning site
°85 a.a. of the Tat protein(starting at a.a.2 and extending to a.a.86)

°A threonine and a serine introduced by cloning procedure
°One glycine and six histidines

Strain Y1737(Mut^s phenotype) producing the recombinant Nef-Tat-His fusion protein, a myristylated 302 amino acids protein which is composed of:

°Myristic acid
°A methionine, created by the use of NcoI cloning site
°205a.a. of Nef protein(starting at a.a.2 and extending to a.a.206)
°A threonine and a serine created by the cloning procedure
°85a.a. of the Tat protein(starting at a.a.2 and extending to a.a.86)
°A threonine and a serine introduced by the cloning procedure
°One glycine and six histidines

Example 2. EXPRESSION OF HIV-1 Tat-MUTANT IN PICHIA PASTORIS

A mutant recombinant Tat protein has also been expressed. The mutant Tat protein must be **biologically inactive** while **maintaining** its **immunogenic epitopes.**

A double mutant *tat* gene, constructed by D.Clements (Tulane University) was selected for these constructs.

This *tat* gene (originates from BH10 molecular clone) bears **mutations** in the **active site region (Lys41→Ala)**and in **RGD motif (Arg78→Lys and Asp80→Glu)** (Virology 235: 48-64, 1997).

The mutant *tat* gene was received as a cDNA fragment subcloned between the EcoRI and HindIII sites within a CMV expression plasmid (pCMVLys41/KGE)

2.1 CONSTRUCTION OF THE INTEGRATIVE VECTORS pRIT14912(encoding Tat mutant-His protein) and pRIT14913(encoding fusion Nef-Tat mutant-His).

The *tat* mutant gene was amplified by PCR from the pCMVLys41/KGE plasmid with primers 05 and 04 (see section 1.1construction of pRIT14598)

An NcoI restriction site was introduced at the 5' end of the PCR fragment while a SpeI site was introduced at the 3' end with primer 04. The PCR fragment obtained and the PHIL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose gel and ligated to create the integrative plasmid pRIT14912

To construct pRIT14913, the *tat* mutant gene was amplified by PCR from the pCMVLys41/KGE plasmid with primers 03 and 04.

SpeI

PRIMER 03 (Seq ID NO 3): 5' ATCGTACTAGT.GAG.CCA.GTA.GAT.C 3'

SpeI

PRIMER 04 (Seq ID NO 4): 5' CGGCTACTAGTTTCCTTCGGGCCT 3'

The PCR fragment obtained and the plasmid pRIT14597 (expressing Nef-His protein) were both digested by SpeI restriction enzyme, purified on agarose gel and ligated to create the integrative plasmid pRIT14913

2.2 TRANSFORMATION OF PICHIA PASTORIS STRAIN GS115.

Pichia pastoris strains expressing Tat mutant-His protein and the fusion Nef-Tat mutant-His were obtained, by applying integration and recombinant strain selection strategies previously described in section 1.2 .

Two recombinant strains producing Tat mutant-His protein ,a 95 amino-acids protein, were selected: Y1775 (Mut^+ phenotype) and Y1776(Mut^S phenotype).

One recombinant strain expressing Nef-Tat mutant-His fusion protein, a 302 amino-acids protein was selected: Y1774(Mut^+ phenotype).

Example 3: FERMENTATION OF PICHIA PASTORIS PRODUCING RECOMBINANT TAT-HIS.

A typical process is described in the table hereafter.

Fermentation includes a growth phase (feeding with a glycerol-based medium according to an appropriate curve) leading to a high cell density culture and an induction phase (feeding with a methanol and a salts/micro-elements solution). During fermentation the growth is followed by taking samples and measuring their absorbance at 620 nm. During the induction phase methanol was added via a pump and its concentration monitored by Gas chromatography (on culture samples) and by on-line gas analysis with a Mass spectrometer. After fermentation the cells were recovered by centrifugation at 5020g during 30' at 2-8°C and the cell paste stored at –20°C. For further work cell paste was thawed, resuspended at an OD (at 620 nm) of 150 in a buffer (Na2HPO4 pH7 50 mM, PMSF 5%, Isopropanol 4 mM) and disrupted by 4 passages in a DynoMill (room 0.6L, 3000 rpm, 6L/H, beads diameter of 0.40-0.70 mm).

For evaluation of the expression samples were removed during the induction, disrupted and analyzed by SDS-Page or Western blot. On Coomassie blue stained SDS-gels the recombinant Tat-his was clearly identified as an intense band presenting a maximal intensity after around 72-96H induction.

Thawing of a Working ..eed vial	
↓	
Solid preculture 30°C, 14-16H	Synthetic medium: YNB + glucose + agar
↓	
Liquid preculture in two 2L erlenmeyer 30°C, 200 rpm	Synthetic medium: 2 x 400 ml YNB + glycerol Stop when OD > 1 (at 620 nm)
↓	
Inoculation of a 20L fermentor	5L initial medium (FSC006AA) 3 ml antifoam SAG471 (from Witco) Set-points: Temperature : 30°C Overpressure: 0.3 barg Air flow: 20 Nl/min Dissolved O2: regulated > 40% pH : regulated at 5 by NH_4OH
↓	
Fed-batch fermentation: growth phase Duration around 40H,	Feeding with glycerol-based medium FFB005AA Final OD between 200-500 OD (620 nm)
Fed-batch fermentation: induction phase Duration: up to 97H.	Feeding with methanol and with a salt/micro-elements solution (FSE021AB).
↓	
Centrifugation	5020g /30 min / 2-8°C
↓	
Recover cell paste and store at –20°C	
↓	
Thaw cells and resuspend at OD150 (620 nm) in buffer	Buffer: Na2HPO4 pH7 50 mM, PMSF 5%, Isopropanol 4 mM
↓	
Cell disruption in Dyno-mill 4 passages	Dyno-mill: (room 0.6L, 3000 rpm, 6L/H, beads diameter of 0.40-0.70 mm).
↓	
Transfer for extraction/purification	

Media used for fermentation:

Solid preculture: (YNB + glucose + agar)

Glucose:	10 g/l	Na2MoO4.2H2O:	0.0002 g/l	Acide folique:	0.000064 g/l
KH2PO4:	1 g/l	MnSO4.H2O:	0.0004 g/l	Inositol:	0.064 g/l
MgSO4.7H2O:	0.5 g/l	H3BO3:	0.0005 g/l	Pyridoxine:	0.008 g/l
CaCl2.2H2O:	0.1 g/l	KI:	0.0001 g/l	Thiamine:	0.008 g/l
NaCl:	0.1 g/l	CoCl2.6H2O:	0.00009 g/l	Niacine:	0.000032 g/l
FeCl3.6H2O:	0.0002 g/l	Riboflavine:	0.000016 g/l	Panthoténate Ca:	0.008 g/l
CuSO4.5H2O:	0.00004 g/l	Biotine:	0.000064 g/l	Para-aminobenzoic acid:	0.000016 g/l
ZnSO4.7H2O:	0.0004 g/l	(NH4)2SO4:	5 g/l	Agar	18 g/l

Liquid preculture ,(YNB + glycerol)

Glycerol:	2% (v/v)	Na2MoO4.2H2O:	0.0002 g/l	Acide folique:	0.000064 g/l
KH2PO4:	1 g/l	MnSO4.H2O:	0.0004 g/l	Inositol:	0.064 g/l
MgSO4.7H2O:	0.5 g/l	H3BO3:	0.0005 g/l	Pyridoxine:	0.008 g/l
CaCl2.2H2O:	0.1 g/l	KI:	0.0001 g/l	Thiamine:	0.008 g/l
NaCl:	0.1 g/l	CoCl2.6H2O:	0.00009 g/l	Niacine:	0.000032 g/l
FeCl3.6H2O:	0.0002 g/l	Riboflavine:	0.000016 g/l	Panthoténate Ca:	0.008 g/l
CuSO4.5H2O:	0.00004 g/l	Biotine:	0.000064 g/l	Para-aminobenzoic acid:	0.000016 g/l
ZnSO4.7H2O:	0.0004 g/l	(NH4)2SO4:	5 g/l		

Initial fermentor charge: (FSC006AA)

$(NH4)_2SO4$:	6.4 g/l		
KH2PO4:	9 g/l	Na2MoO4.2H2O:	2.04 mg/l
MgSO4.7H2O:	4.7 g/l	MnSO4.H2O:	4.08 mg/l
CaCl2.2H2O:	0.94 g/l	H3BO3:	5.1 mg/l
FeCl3.6H2O:	10 mg/l	KI:	1.022 mg/l
HCl:	1.67 ml/l	CoCl2.6H2O:	0.91mg/l
CuSO4.5H2O:	0.408 mg/l	NaCl:	0.06 g/l
ZnSO4.7H2O:	4.08 mg/l	Biotine:	0.534 mg/l

Feeding solution used for growth phase (FFB005AA)

Glycérol:	38.7 % v/v	Na2MoO4.2H2O:	5.7 mg/l
MgSO4.7H2O:	13 g/l	CuSO4.5H2O:	1.13 mg/l
CaCl2.2H2O:	2.6 g/l	CoCl2.6H2O:	2.5 mg/l
FeCl3.6H2O:	27.8mg/l	H3BO3:	14.2 mg/l
ZnSO4.7H2O	11.3 mg/l	Biotine:	1.5 mg/l
MnSO4.H2O:	11.3 mg/l	KI:	2.84mg/l
KH2PO4:	24.93 g/l	NaCl:	0.167 g/l

Feeding solution of salts and micro-elements used during induction (FSE021AB):

KH2PO4:	45 g/l	Na2MoO4.2H2O:	10.2 mg/l
MgSO4.7H2O:	23.5 g/l	MnSO4.H2O:	20.4 mg/l
CaCl2.2H2O:	4.70 g/l	H3BO3:	25.5 mg/l
NaCl:	0.3 g/l	KI:	5.11 mg/l
HCl:	8.3 ml/l	CoCl2.6H2O:	4.55mg/l
CuSO4.5H2O:	2.04 mg/l	FeCl3.6H2O:	50.0 mg/l
ZnSO4.7H2O:	20.4 mg/l	Biotine:	2.70 mg/l

Example 4: PURIFICATION OF Nef-Tat-His FUSION PROTEIN (PICHIA PASTORIS)

The purification scheme has been developed from 146g of recombinant Pichia pastoris cells (wet weight) or 2L Dyno-mill homogenate OD 55. The chromatographic steps are performed at room temperature. Between steps , Nef-Tat positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

146g of Pichia pastoris cells	
↓	
Homogenization	Buffer: 2L 50 mM PO_4 pH 7.0 final OD:50
↓	
Dyno-mill disruption (4 passes)	
↓	
Centrifugation	JA10 rotor / 9500 rpm/ 30 min / room temperature
↓	
Dyno-mill Pellet	
↓	
Wash (1h - 4°C)	Buffer: +2L 10 mM PO_4 pH 7.5 - 150mM - NaCl 0,5% empigen
↓	
Centrifugation	JA10 rotor / 9500 rpm/ 30 min / room temperature
↓	

Pellet	
↓	
Solubilisation (O/N - 4°C)	Buffer: + 660ml 10 mM PO_4 pH 7.5 - 150mM NaCl - 4.0M GuHCl
↓	
Reduction (4H – room temperature - in the dark)	+ 0,2M 2-mercaptoethanesulfonic acid, sodium salt (powder addition) / pH adjusted to 7.5 (with 0,5M NaOH solution) before incubation
↓	
carbamidomethylation (1/2 h – room temperature - in the dark)	+ 0,25M Iodoacetamide (powder addition) / pH adjusted to 7.5 (with 0,5M NaOH solution) before incubation
↓	
Immobilized metal ion affinity chromatography on Ni^{++}-NTA-Agarose (Qiagen - 30 ml of resin)	Equilibration buffer: 10 mM PO_4 pH 7.5 - 150mM NaCl - 4.0M GuHCl Washing buffer: 1) Equilibration buffer 2) 10 mM PO_4 pH 7.5 - 150mM NaCl - 6M Urea 3) 10 mM PO_4 pH 7.5 - 150mM NaCl - 6M Urea - 25 mM Imidazol Elution buffer: 10 mM PO_4 pH 7.5 - 150mM NaCl - 6M Urea - 0,5M Imidazol

↓	
Dilution	Down to an ionic strength of 18 mS/cm²
	Dilution buffer: 10 mM PO_4 pH 7.5 - 6M Urea
↓	
Cation exchange chromatography on SP Sepharose FF	Equilibration buffer: 10 mM PO_4 pH 7.5 - 150mM NaCl - 6.0M Urea
(Pharmacia - 30 ml of resin)	Washing buffer: 1) Equilibration buffer
	2) 10 mM PO_4 pH 7.5 - 250mM NaCl - 6M Urea
	Elution buffer: 10 mM Borate pH 9.0 - 2M NaCl - 6M Urea
↓	
Concentration	up to 5 mg/ml
	10kDa Omega membrane(Filtron)
↓	
Gel filtration chromatography on Superdex200 XK 16/60	Elution buffer: 10 mM PO_4 pH 7.5 - 150mM NaCl - 6M Urea
(Pharmacia - 120 ml of resin)	5 ml of sample / injection → 5 injections
↓	
Dialysis	Buffer: 10 mM PO_4 pH 6.8 -
(O/N - 4°C)	150mM NaCl - 0,5M Arginin*
↓	
Sterile filtration	Millex GV 0,22µm

* ratio: 0,5M Arginin for a protein concentration of 1600µg/ml.

Purity

The level of purity as estimated by SDS-PAGE is shown in Figure 3 by Daiichi Silver Staining and in Figure 4 by Coomassie blue G250.

After Superdex200 step:	> 95%
After dialysis and sterile filtration steps:	> 95%

Recovery

51mg of Nef-Tat-his protein are purified from 146g of recombinant Pichia pastoris cells (= 2L of Dyno-mill homogenate OD 55)

Example 5: PURIFICATION OF OXIDIZED NEF-TAT-HIS FUSION PROTEIN IN PICHIA PASTORIS

The purification scheme has been developed from 73 g of recombinant Pichia pastoris cells (wet weight) or 1 L Dyno-mill homogenate OD 50. The chromatographic steps are performed at room temperature. Between steps , Nef-Tat positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

73 g of Pichia pastoris cells

↓

Homogenization — Buffer: 1L 50 mM PO_4 pH 7.0 – Pefabloc 5 mM; final OD:50

↓

Dyno-mill disruption (4 passes)

↓

Centrifugation	JA10 rotor / 9500 rpm/ 30 min / room temperature
↓	
Dyno-mill Pellet	
↓	
Wash (2h - 4°C)	Buffer: +1L 10 mM PO_4 pH 7.5 – 150 mM NaCl - 0,5% Empigen
↓	
Centrifugation	JA10 rotor / 9500 rpm/ 30 min / room temperature
↓	
Pellet	
↓	
Solubilisation (O/N - 4°C)	Buffer: + 330ml 10 mM PO_4 pH 7.5 - 150mM NaCl - 4.0M GuHCl
↓	
Immobilized metal ion affinity chromatography on Ni^{++}-NTA-Agarose (Qiagen - 15 ml of resin)	Equilibration buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl - 4.0 M GuHCl Washing buffer: 1) Equilibration buffer 2) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea 3) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 25 mM Imidazol Elution buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 0,5 M Imidazol
↓	
Dilution	Down to an ionic strength of 18 mS/cm² Dilution buffer: 10 mM PO_4 pH 7.5 – 6 M Urea
↓	
Cation exchange chromatography on SP Sepharose FF (Pharmacia - 7 ml of resin)	Equilibration buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl - 6.0 M Urea Washing buffer: 1) Equilibration buffer 2) 10 mM PO_4 pH 7.5 – 250 mM NaCl – 6 M Urea

	Elution buffer: 10 mM Borate pH 9.0 – 2 M NaCl – 6 M Urea
↓	
Concentration	up to 0,8 mg/ml 10kDa Omega membrane(Filtron)
↓	
Dialysis **(O/N - 4°C)**	Buffer: 10 mM PO_4 pH 6.8 – 150 mM NaCl – 0,5 M Arginin
↓	
Sterile filtration	Millex GV 0,22µm

➔ Level of purity estimated by SDS-PAGE is shown in Figure 6 (Daiichi Silver Staining, Coomassie blue G250, Western blotting):

After dialysis and sterile filtration steps: > 95%

➔ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

2,8 mg of oxidized Nef-Tat-his protein are purified from 73 g of recombinant Pichia pastoris cells (wet weight) or 1 L of Dyno-mill homogenate OD 50.

Example 6: PURIFICATION OF REDUCED TAT-HIS PROTEIN (PICHIA PASTORIS)

The purification scheme has been developed from 160 g of recombinant Pichia pastoris cells (wet weight) or 2L Dyno-mill homogenate OD 66. The chromatographic steps are performed at room temperature. Between steps, Tat positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

160 g of Pichia pastoris cells

↓

Homogenization — Buffer: +2 L 50 mM PO_4 pH 7.0 – 4 mM PMSF final OD:66

↓

Dyno-mill disruption (4 passes)

↓

Centrifugation — JA10 rotor / 9500 rpm / 30 min / room temperature

↓

Dyno-mill Pellet

↓

Wash (1h - 4°C) — Buffer: +2 L 10 mM PO_4 pH 7.5 – 150 mM NaCl - 1% Empigen

↓

Centrifugation — JA10 rotor / 9500 rpm / 30 min / room temperature

↓

Pellet

↓

Solubilisation (O/N - 4°C) — Buffer: + 660 ml 10 mM PO_4 pH 7.5 – 150 mM NaCl - 4.0 M GuHCl

↓

Centrifugation — JA10 rotor / 9500 rpm / 30 min / room temperature

↓

Reduction (4H - room temperature - in the dark) — + 0,2 M 2-mercaptoethanesulfonic acid, sodium salt (powder addition) / pH adjusted to 7.5 (with 1 M NaOH solution) before incubation

↓

carbamidomethylation (1/2 h - room temperature - in the dark) — + 0,25 M Iodoacetamide (powder addition) / pH adjusted to 7.5 (with 1 M NaOH solution) before incubation

↓

Immobilized metal ion affinity chromatography on Ni^{++}-NTA-Agarose (Qiagen - 60 ml of resin)

Equilibration buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl - 4.0 M GuHCl

Washing buffer: 1) Equilibration buffer

2) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea

3) 10 mM PO_4 pH 7.5 – 150 mM

	NaCl - 6M Urea - 35 mM Imidazol
	Elution buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 0,5 M Imidazol
↓	
Dilution	Down to an ionic strength of 12 mS/cm
	Dilution buffer: 20 mM Borate pH 8.5 – 6 M Urea
↓	
Cation exchange chromatography on SP Sepharose FF	Equilibration buffer: 20 mM Borate pH 8.5 - 150 mM NaCl - 6.0 M Urea
(Pharmacia - 30 ml of resin)	Washing buffer: Equilibration buffer
	Elution buffer: 20 mM Borate pH 8.5 – 400 mM NaCl - 6.0 M Urea
↓	
Concentration	up to 1,5 mg/ml
	10kDa Omega membrane(Filtron)
↓	
Dialysis (O/N - 4°C)	Buffer: 10 mM PO_4 pH 6.8 – 150 mM NaCl - 0,5 M Arginin
↓	
Sterile filtration	Millex GV 0,22 µm

→ Level of purity estimated by SDS-PAGE as shown in Figure 7(Daiichi Silver Staining, Coomassie blue G250, Western blotting):

After dialysis and sterile filtration steps: > 95%

→ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

48 mg of reduced Tat-his protein are purified from 160 g of recombinant Pichia pastoris cells (wet weight) or 2 L of Dyno-mill homogenate OD 66.

Example 7: Purification of oxidized Tat-his protein (Pichia Pastoris)

The purification scheme has been developed from 74 g of recombinant Pichia pastoris cells (wet weight) or 1L Dyno-mill homogenate OD60. The chromatographic steps are performed at room temperature. Between steps, Tat positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

74 g of Pichia pastoris cells	
↓	
Homogenization	Buffer: +1 L 50 mM PO_4 pH 7.0 – 5 mM Pefabloc final OD:60
↓	
Dyno-mill disruption (4 passes)	
↓	
Centrifugation	JA10 rotor / 9500 rpm / 30 min / room temperature
↓	
Dyno-mill Pellet	
↓	
Wash (1h - 4°C)	Buffer:+1 L 10 mM PO_4 pH 7.5 – 150 mM NaCl - 1% Empigen
↓	
Centrifugation	JA10 rotor / 9500 rpm / 30 min / room temperature
↓	
Pellet	
↓	
Solubilisation (O/N - 4°C)	Buffer: + 330 ml 10 mM PO_4 pH 7.5 – 150 mM NaCl - 4.0 M GuHCl
↓	
Centrifugation	JA10 rotor / 9500 rpm / 30 min / room temperature
↓	

Immobilized metal ion affinity chromatography on Ni^{++}-NTA-Agarose (Qiagen - 30 ml of resin)	Equilibration buffer: 10 mM PO_4 pH 7.5 –150 mM NaCl - 4.0 M GuHCl Washing buffer: 1) Equilibration buffer 2) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea 3) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 35 mM Imidazol Elution buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 0,5 M Imidazol
↓	
Dilution	Down to an ionic strength of 12 mS/cm Dilution buffer: 20 mM Borate pH 8.5 – 6 M Urea
↓	
Cation exchange chromatography on SP Sepharose FF (Pharmacia - 15 ml of resin)	Equilibration buffer: 20 mM Borate pH 8.5 - 150 mM NaCl - 6.0 M Urea Washing buffer: 1) Equilibration buffer 2) 20 mM Borate pH 8.5 - 400 mM NaCl - 6.0 M Urea Elution buffer: 20 mM Piperazine pH 11.0 – 2 M NaCl – 6 M Urea
↓	
Concentration	up to 1,5 mg/ml 10 kDa Omega membrane(Filtron)
↓	
Dialysis (O/N - 4°C)	Buffer: 10 mM PO_4 pH 6.8 – 150 mM NaCl - 0,5 M Arginin
↓	
Sterile filtration	Millex GV 0,22 µm

→ Level of purity estimated by SDS-PAGE as shown in Figure 8 (Daiichi Silver Staining, Coomassie blue G250, Western blotting):

After dialysis and sterile filtration steps: > 95%

→ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

19 mg of oxidized Ta:-his protein are purified from 74 g of recombinant Pichia pastoris cells (wet weight) or 1 L of Dyno-mill homogenate OD 60.

Example 8: PURIFICATION OF SIV REDUCED NEF-HIS PROTEIN (PICHIA PASTORIS)

The purification scheme has been developed from 340 g of recombinant Pichia pastoris cells (wet weight) or 4 L Dyno-mill homogenate OD 100. The chromatographic steps are performed at room temperature. Between steps , Nef positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

340 g of Pichia pastoris cells	
↓	
Homogenization	Buffer: 4L 50 mM PO_4 pH 7.0 – PMSF 4 mM final OD:100
↓	
Dyno-mill disruption (4 passes)	
↓	
Centrifugation	JA10 rotor / 9500 rpm/ 60 min / room temperature
↓	
Dyno-mill Pellet	
↓	
Solubilisation (O/N - 4°C)	Buffer: + 2,6 L 10 mM PO_4 pH 7.5 - 150mM NaCl - 4.0M GuHCl
↓	
Centrifugation	JA10 rotor / 9500 rpm / 30 min / room temperature
↓	
Reduction (4H - room temperature - in the dark)	+ 0,2 M 2-mercaptoethanesulfonic acid, sodium salt (powder addition) / pH adjusted to 7.5 (with

	1 M NaOH solution) before incubation
↓	
Carbamidomethylation **(1/2 h - room temperature - in the dark)**	+ 0,25 M Iodoacetamide (powder addition) / pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
Immobilized metal ion affinity chromatography on Ni^{++}-NTA-Agarose (Qiagen - 40 ml of resin)	Equilibration buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl - 4.0 M GuHCl Washing buffer: 1) Equilibration buffer 2) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 25 mM Imidazol Elution buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 0,5 M Imidazol
↓	
Concentration	up to 3 mg/ml 10kDa Omega membrane(Filtron)
↓	
Gel filtration chromatography on Superdex 200 **(Pharmacia - 120 ml of resin)**	Elution buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea
↓	
Concentration	up to 1,5 mg/ml 10kDa Omega membrane(Filtron)
↓	
Dialysis **(O/N - 4°C)**	Buffer: 10 mM PO_4 pH 6.8 – 150 mM NaCl – Empigen 0,3%
↓	
Sterile filtration	Millex GV 0,22µm

➔ Level of purity estimated by SDS-PAGE as shown in Figure 9 (Daiichi Silver Staining, Coomassie blue G250, Western blotting):

After dialysis and sterile filtration steps: > 95%

➔ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

20 mg of SIV reduced Nef -his protein are purified from 340 g of recombinant Pichia pastoris cells (wet weight) or 4 L of Dyno-mill homogenate OD 100.

Example 9: PURIFICATION OF HIV REDUCED NEF-HIS PROTEIN (PICHIA PASTORIS)

The purification scheme has been developed from 160 g of recombinant Pichia pastoris cells (wet weight) or 3 L Dyno-mill homogenate OD 50. The chromatographic steps are performed at room temperature. Between steps , Nef positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

160 g of Pichia pastoris cells	
↓	
Homogenization	Buffer: 3 L 50 mM PO_4 pH 7.0 – Pefabloc 5 mM final OD:50
↓	
Dyno-mill disruption (4 passes)	
↓	
Freezing/Thawing	
↓	
Centrifugation	JA10 rotor / 9500 rpm/ 60 min / room temperature
↓	
Dyno-mill Pellet	
↓	
Solubilisation (O/N - 4°C)	Buffer: + 1 L 10 mM PO_4 pH 7.5 - 150mM NaCl - 4.0M GuHCl
↓	
Centrifugation	JA10 rotor / 9500 rpm / 60 min / room temperature
↓	

Reduction (3 H - room temperature - in the dark)	+ 0,1 M 2-mercaptoethanesulfonic acid, sodium salt (powder addition) / pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
Carbamidomethylation (1/2 h - room temperature - in the dark)	+ 0,15 M Iodoacetamide (powder addition) / pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
Immobilized metal ion affinity chromatography on Ni^{++}-NTA-Agarose (Qiagen - 10 ml of resin)	Equilibration buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl - 4.0 M GuHCl Washing buffer: 1) Equilibration buffer 2) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea 3) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 25 mM Imidazol Elution buffer: 10 mM Citrate pH 6.0 - 150 mM NaCl – 6 M Urea - 0,5 M Imidazol
↓	
Concentration	up to 3 mg/ml 10kDa Omega membrane(Filtron)
↓	
Gel filtration chromatography on Superdex 200 (Pharmacia - 120 ml of resin)	Elution buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea
↓	
Dialysis (O/N - 4°C)	Buffer: 10 mM PO_4 pH 6.8 – 150 mM NaCl – 0,5M Arginin
↓	
Sterile filtration	Millex GV 0,22µm

→ Level of purity estimated by SDS-PAGE as shown in Figure 10 (Daiichi Silver Staining, Coomassie blue G250, Western blotting):

After dialysis and sterile filtration steps: > 95%

→ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

20 mg of HIV reduced Nef-his protein are purified from 160 g of recombinant Pichia pastoris cells (wet weight) or 3 L of Dyno-mill homogenate OD 50.

Example 10: EXPRESSION OF SIV *nef* SEQUENCE IN *PICHIA PASTORIS*

In order to evaluate Nef and Tat antigens in the pathogenic SHIV challenge model, we have expressed the Nef protein of simian immunodeficiency virus (SIV) of macaques, SIVmac239 (Aids Research and Human Retroviruses, 6:1221-1231,1990).
In the Nef coding region , SIV mac 239 has an in-frame stop codon after 92aa predicting a truncated product of only 10kD. The remainder of the Nef reading frame is open and would be predicted to encode a protein of 263aa (30kD) in its fully open form.

Our starting material for SIVmac239 *nef* gene was a DNA fragment corresponding to the complete coding sequence, cloned on the LX5N plasmid (received from Dr R.C. Desrosiers, Southborough,MA,USA) .
This SIV *nef* gene is mutated at the premature stop codon (nucleotide G at position 9353 replaces the original T nucleotide) in order to express the full-length SIVmac239 Nef protein.

To express this SIV *nef* gene in *Pichia pastoris*, the PHIL-D2-MOD Vector (previously used for the expression of HIV-1 *nef* and *tat* sequences) was used. The recombinant protein is expressed under the control of the inducible alcohol oxidase (AOX1) promoter and the c-terminus of the protein is elongated by a Histidine affinity tail that will facilitate the purification.

10.1 CONSTRUCTION OF THE INTEGRATIVE VECTOR pRIT 14908

To construct **pRIT 14908** , the SIV *nef* gene was amplified by PCR from the pLX5N/SIV-NEF plasmid with primers SNEF1 and SNEF2.

PRIMER SNEF1: 5' ATCGTCCATG.GGTGGAGCTATTTT 3'
NcoI

PRIMER SNEF2: 5' CGGCTACTAGTGCGAGTTTCCTT 3'
SpeI

The SIV *nef* DNA region amplified starts at nucleotide 9077 and terminates at nucleotide 9865 (Aids Research and Human Retroviruses, 6:1221-1231,1990).

An NcoI restriction site (with carries the ATG codon of the *nef* gene) was introduced at the 5' end of the PCR fragment while a SpeI site was introduced at the 3' end. The PCR fragment obtained and the integrative PHIL-D2-MOD vector were both restricted by NcoI and SpeI. Since one NcoI restriction site is present on the SIV *nef* amplified sequence (at position 9286), two fragments of respectively ±200bp and ± 600bp were obtained, purified on agarose gel and ligated to PHIL-D2-MOD vector. The resulting recombinant plasmid received, after verification of the *nef* amplified region by automated sequencing, the pRIT 14908 denomination.

10.2 TRANSFORMATION OF PICHIA PASTORIS STRAIN GS115(his4).

To obtain *Pichia pastoris* strain expressing SIV *nef*-His, strain GS115 was transformed with a linear NotI fragment carrying only the expression cassette and the HIS4 gene (Fig.11).
This linear NotI DNA fragment ,with homologies at both ends with AOX1 resident *P.pastoris* gene, favors recombination at the AOX1 locus.
Multicopy integrant clones were selected by quantitative dot blot analysis .
One transformant showing the best production level for the recombinant protein was selected and received the **Y1772** denomination.

Strain **Y1772** produces the recombinant **SIV Nef-His** protein, a **272 amino acids** protein which would be composed of:
°Myristic acid
°A methionine, created by the use of NcoI cloning site of PHIL-D2-MOD vector .

°262 amino acids (aa) of Nef protein (starting at aa 2 and extending to aa 263, see Figure 12)
°A threonine and a serine created by the cloning procedure (cloning at SpeI site of PHIL-D2-MOD vector (Fig.11).
°One glycine and six histidines.

Nucleic and Protein sequences are shown on figure 12.

10.3 CHARACTERIZATION OF THE EXPRESSED PRODUCT OF STRAIN Y1772.

Expression level

After 16 hours induction in medium containing 1% methanol as carbon source, abundance of the recombinant Nef-His protein, was estimated at **10%** of total protein (Fig.13 , lanes 3-4).

Solubility

Induced cultures of recombinant strain Y1772 producing the Nef-His protein were centrifuged. Cell pellets were resuspended in breaking buffer, disrupted with 0.5mm glass beads and the cell extracts were centrifuged. The proteins contained in the insoluble pellet (P) and in the soluble supernatant (S) were compared on a Coomassie Blue stained SDS-PAGE10%.
As shown in figure 13, the majority of the recombinant protein from strain Y1772 (lanes 3-4) is associated with the insoluble fraction.

Strain Y1772 which presents a satisfactory recombinant protein expression level is used for the production and purification of SIV Nef-His protein.

Example 11: EXPRESSION OF GP120 IN CHO

A stable CHO-K1 cell line which produces a recombinant gP120 glycoprotein has been established. Recombinant gP120 glycoprotein is a recombinant truncated form of the gP120 envelope protein of HIV-1 isolate W61D. The protein is excreted into the cell culture medium, from which it is subsequently purified.

Construction of gp120 transfection plasmid pRIT13968

The envelope DNA coding sequence (including the 5'exon of tat and rev) of HIV-1 isolate W61D was obtained (Dr. Tersmette, CCB, Amsterdam) as a genomic gp160 envelope containing plasmid W61D (Nco-XhoI). The plasmid was designated pRIT13965.

In order to construct a gp120 expression cassette a stop codon had to be inserted at the amino acid glu 515 codon of the gp160 encoding sequence in pRIT13965 using a primer oligonucleotide sequence (DIR 131) and PCR technology. Primer DIR 131 contains three stop codons (in all open reading frames) and a SalI restriction site.

The complete gp120 envelope sequence was then reconstituted from the N-terminal BamH1-DraI fragment (170 bp) of a gp160 plasmid subclone pW61d env (pRIT13966) derived from pRIT13965, and the DraI-SalI fragment (510 bp) generated by PCR from pRIT13965. Both fragments were gel purified and ligated together into the E.coli plasmid pUC18, cut first by SalI (klenow treated), and then by BamH1. This resulted in plasmid pRIT13967. The gene sequence of the XmaI-SalI fragment (1580 bp) containing the gp120 coding cassette was sequenced and found to be identical to the predicted sequence. Plasmid RIT13967 was ligated into the CHO GS-expression vector pEE14 (Celltech Ltd., UK) by cutting first with BclI (klenow treated) and then by XmaI. The resulting plasmid was designated pRIT13968.

Preparation of Master Cell Bank

The gp120-construct (pRIT13968) was transfected into CHO cells by the classical $CaPO_4$-precipitation/glycerol shock procedure. Two days later the CHOK1 cells were subjected to selective growth medium (GMEM + methionine sulfoximine (MSX) 25 µM + Glutamate + asparagine + 10% Foetal calf serum). Three chosen

transfectant clones were further amplified in 175m² flasks and few cell vials were stored at -80°C. C-env 23,9 was selected for further expansion.

A small prebank of cells was prepared and 20 ampoules were frozen. For preparation of the prebank and the MCB, cells were grown in GMEM culture medium, supplemented with 7.5 % fetal calf serum and containing 50 µM MSX. These cell cultures were tested for sterility and mycoplasma and proved to be negative.

The Master Cell Bank CHOK1 env 23.9 (at passage 12) was prepared using cells derived from the premaster cell bank. Briefly, two ampoules of the premaster seed were seeded in medium supplemented with 7.5% dialysed foetal bovine serum. The cells were distributed in four culture flasks and cultured at 37°C. After cell attachment the culture medium was changed with fresh medium supplemented with 50 µM MSX. At confluence, cells were collected by trypsination and subcultured with a 1/8 split ratio in T-flasks - roller bottle - cell factory units. Cells were collected from cell factory units by trypsination and centrifugation. The cell pellet was resuspended in culture medium supplemented with DMSO as cryogenic preservative. Ampoules were prelabelled, autoclaved and heat-sealed (250 vials). They were checked for leaks and stored overnight at -70°C before storage in liquid nitrogen.

Cell Culture And Production Of Crude Harvest

Two vials from a master cell bank are thawed rapidly. Cells are pooled and inoculated in two T-flasks at 37° ± 1°C with an appropriate culture medium supplemented with 7.5 % dialysed foetal bovine (FBS) serum. When reaching confluence (passage 13), cells are collected by trypsinisation, pooled and expanded in 10 T-flasks as above. Confluent cells (passage 14) are trypsinised and expanded serially in 2 cell factory units (each 6000 cm²; passage 15), then in 10 cell factories (passage 16). The growth culture medium is supplemented with 7.5 % dialysed foetal bovine (FBS) serum and 1% MSX. When cells reach confluence, the growth culture medium is discarded and replaced by "production medium" containing only 1 % dialysed foetal bovine serum and no MSX. Supernatant is collected every two

days (48 hrs-interval) for up to 32 days. The harvested culture fluids are clarified immediately through a 1.2-0.22 µm filter unit and kept at -20°C before purification.

Example 12: PURIFICATION OF HIV GP 120 (W61D CHO) FROM CELL CULTURE FLUID

All purification steps are performed in a cold room at 2-8°C. pH of buffers are adjusted at this temperature and are filtered on 0.2 µm filter. They are tested for pyrogen content (LAL assay). Optical density at 280 nm, pH and conductivity of column eluates are continuously monitored.

(i) Clarified Culture Fluid

The harvested clarified cell culture fluid (CCF) is filter-sterilized and Tris buffer, pH 8.0 is added to 30 mM final concentration. CCF is stored frozen at -20°C until purification.

(ii) Hydrophobic Interaction Chromatography

After thawing, ammonium sulphate is added to the clarified culture fluid up to 1 M. The solution is passed overnight on a TSK/TOYOPEARL-BUTYL 650 M (TOSOHAAS) column, equilibrated in 30 mM Tris buffer- pH 8.0 - 1 M ammonium sulphate. Under these conditions, the antigen binds to the gel matrix. The column is washed with a decreasing stepwise ammonium sulphate gradient. The antigen is eluted at 30 mM Tris buffer- pH 8.0 - 0.25 M ammonium sulphate.

(iii) Anion-exchange Chromatography

After reducing the conductivity of the solution between 5 and 6 mS/cm, the gP120 pool of fractions is loaded onto a Q-sepharose Fast Flow (Pharmacia) column, equilibrated in Tris-saline buffer - pH 8.0. The column is operated on a negative mode, i.e. gP120 does not bind to the gel, while most of the impurities are retained.

(iv) Concentration and diafiltration by ultrafiltration

In order to increase the protein concentration, the gP120 pool is loaded on a FILTRON membrane "Omega Screen Channel", with a 50 kDa cut-off. At the end of the concentration, the buffer is exchanged by diafiltration with 5 mM phosphate

buffer containing $CaCl_2$ 0.3 mM, pH 7.0. If further processing is not performed immediately, the gP120 pool is stored frozen at -20°C. After thawing the solution is filtered onto a 0.2 µM membrane in order to remove insoluble materiel.

(v) Chromatography on hydroxyapatite

The gP120 UF pool is loaded onto a macro-Prep Ceramic Hydroxyapatite, type II (Biorad) column equilibrated in 5 mM phosphate buffer + $CaCl_2$ 0.3 mM, pH 7.0. The column is washed with the same buffer. The antigen passes through the column and impurities bind to the column.

(vi) Cation exchange chromatography

The gP120 pool is loaded on a CM/TOYOPEARL-650 S (TOSOHAAS) column equilibrated in acetate buffer 20 mM, pH 5.0. The column is washed with the same buffer, then acetate 20 mM, pH 5.0 and NaCl 10 mM. The antigen is then eluted by the same buffer containing 80 mM NaCl.

(vii) Ultrafiltration

In order to augment the virus clearance capacity of the purification process, an additional ultrafiltration step is carried out. The gP120 pool is subjected to ultrafiltration onto a FILTRON membrane "Omega Screen Channel", cut-off 150 kDa. This pore-size membrane does not retain the antigen. After the process, the diluted antigen is concentrated on the same type of membrane (Filtron) but with a cut-off of 50 kDa.

(viii) Size exclusion Gel Chromatography

The gP120 pool is applied to a SUPERDEX 200 (PHARMACIA) column in order to exchange the buffer and to eliminate residual contaminants. The column is eluted with phosphate buffer saline (PBS).

(ix) Sterile filtration and storage

Fractions are sterilized by filtration on a 0.2 µM PVDF membrane (Millipore). After sterile filtration, the purified bulk is stored frozen at -20°C up to formulation. The purification scheme is summarized by the flow sheet below.

⇒ Level of purity of the purified bulk estimated by SDS-PAGE analysis (Silver staining / Coomassie Blue / Western Blotting) is ≥ 95%.

⇒ Production yield is around 2.5 mg /L CCF (according to Lowry assay) - Global purification yield is around 25% (according to Elisa assay)

⇒ Purified material is stable 1 week at 37°C (according to WB analysis)

Purification of gp120 from culture fluid

Mark √ indicate steps that are critical for virus removal.

CLARIFIED CULTURE FLUID
↓
HYDROPHOBIC INTERACTION CHROMATOGRAPHY
(BUTYL -TOYOPEARL 650 M)
↓
ANION EXCHANGE CHROMATOGRAPHY √
(NEGATIVE MODE)
(Q-SEPHAROSE)
↓
50 KD ULTRAFILTRATION
(CONCENTRATION AND BUFFER EXCHANGE)
↓
(STORAGE -20°C)
↓
HYDROXYAPATITE CHROMATOGRAPHY
(NEGATIVE MODE)
(MACROPREP CERAMIC HYDROXYAPATITE II)
↓
CATION EXCHANGE CHROMATOGRAPHY
(CM-TOYOPEARL 650 S)
↓
150 KD ULTRAFILTRATION √
(OMEGA MEMBRANES / FILTRON)
↓

50 KD ULTRAFILTRATION
(CONCENTRATION)
↓

SIZE EXCLUSION CHROMATOGRAPHY √
(SUPERDEX 200)
STERILE FILTRATION
↓

PURIFIED BULK
STORAGE -20°C

Example 13: VACCINE PREPARATION

A vaccine prepared in accordance with the invention comprises the expression products of one or more DNA recombinants encoding an antigen. Furthermore, the formulations comprise a mixture of 3 de -O-acylated monophosphoryl lipid A 3D-MPL and QS21 in an oil/water emulsion or an oligonucleotide containing unmethylated CpG dinucleotide motifs and aluminium hydroxide as carrier.

3D-MPL: is a chemically detoxified form of the lipopolysaccharide (LPS) of the Gram-negative bacteria Salmonella minnesota.

Experiments performed at Smith Kline Beecham Biologicals have shown that 3D-MPL combined with various vehicles strongly enhances both the humoral immunity and a T_{HI} type of cellular immunity.

QS21: is a saponin purified from a crude extract of the bark of the Quillaja Saponaria Molina tree, which has a strong adjuvant activity: it induces both antigen-specific lymphoproliferation and CTLs to several antigens.
Experiments performed at Smith Kline Beecham Biologicals have demonstrated a clear synergistic effect of combinations of 3D-MPL and QS21 in the induction of both humoral and T_{HI} type cellular immune responses.

The oil/water emulsion is composed of 2 oils (a tocopherol and squalene), and of PBS containing Tween 80 as emulsifier. The emulsion comprises 5% squalene, 5%

tocopherol, 2% Tween 80 and has an average particle size of 180 nm (see WO 95/17210).

Experiments performed at Smith Kline Beecham Biologicals have proven that the adjunction of this O/W emulsion to 3D-MPL/QS21 further increases their immunostimulant properties.

Preparation of the oil/water emulsion (2 fold concentrate)

Tween 80 is dissolved in phosphate buffered saline (PBS) to give a 2% solution in the PBS. To provide 100ml two fold concentrate emulsion 5g of DL alpha tocopherol and 5ml of squalene are vortexed to mix thoroughly. 90ml of PBS/Tween solution is added and mixed thoroughly. The resulting emulsion is then passed through a syringe and finally microfluidised by using an M110S Microfluidics machine. The resulting oil droplets have a size of approximately 180 nm.

Preparation of oil in water formulation.

Antigens (100 μg gp120, 20 μg NefTat, and 20 μg SIV Nef, alone or in combination) were diluted in 10 fold concentrated PBS pH 6.8 and H_2O before consecutive addition of the oil in water emulsion, 3D-MPL (50μg), QS21 (50μg) and 1 μg/ml thiomersal as preservative at 5 min interval. The emulsion volume is equal to 50% of the total volume (250μl for a dose of 500μl).

All incubations were carried out at room temperature with agitation.

CpG oligonucleotide (CpG) is a synthetic unmethylated oligonucleotide containing one or several CpG sequence motifs. CpG is a very potent inducer of T_{H1} type immunity compared to the oil in water formulation that induces mainly a mixed T_{H1}/T_{H2} response. CpG induces lower level of antibodies than the oil in water formulation and a good cell mediated immune response. CpG is expected to induce lower local reactogenicity.

Preparation of CpG oligonucleotide solution: CpG dry powder is dissolved in H_2O to give a solution of 5 mg/ml CpG.

Preparation of CpG formulation.

The 3 antigens were dialyzed against NaCl 150 mM to eliminate the phosphate ions that inhibit the adsorption of gp120 on aluminium hydroxide.

The antigens diluted in H_2O (100 µg gp120, 20 µg NefTat and 20 µg SIV Nef) were incubated with the CpG solution (500 µg CpG) for 30 min before adsorption on $Al(OH)_3$ to favor a potential interaction between the His tail of NefTat and Nef antigens and the oligonucleotide (stronger immunostimulatory effect of CpG described when bound to the antigen compared to free CpG). Then were consecutively added at 5 min interval $Al(OH)_3$ (500 µg), 10 fold concentrated NaCl and 1 µg/ml thiomersal as preservative.

All incubations were carried out at room temperature with agitation.

Example 14: IMMUNIZATION AND SHIV CHALLENGE EXPERIMENT IN RHESUS MONKEYS.

<u>First Study</u>

Groups of 4 rhesus monkeys were immunized intramuscularly at 0, 1 and 3 months with the following vaccine compositions:

Group 1:	Adjuvant 2	+ gp120		
Group 2:	Adjuvant 2	+ gp120	+ NefTat	+ SIV Nef
Group 3:	Adjuvant 2		+ NefTat*	+ SIV Nef
Group 4	Adjuvant 6	+ gp120	+ NefTat	+ SIV Nef
Group 5	Adjuvant 2		+ NefTat	+ SIV Nef
Group 6	Adjuvant 2			

Adjuvant 2 comprises squalene/tocopherol/Tween 80/3D-MPL/QS21 and Adjuvant 6 comprises alum and CpG.

Tat* represents mutated Tat, in which Lys41→Ala and in RGD motif Arg78→Lys and Asp80→Glu (Virology 235: 48-64, 1997).

One month after the last immunization all animals were challenged with a pathogenic SHIV (strain 89.6p). From the week of challenge (wk16) blood samples were taken periodically at the indicated time points to determine the % of CD4-positive cells among peripheral blood mononuclear cells by FACS analysis (Figure 14) and the concentration of RNA viral genomes in the plasma by bDNA assay (Figure 15).

Results

All animals become infected after challenge with $SHIV_{89.6p}$.

CD4-positive cells decline after challenge in all animals of groups 1, 3, 5 and 6 except one animal in each of groups 1 and 6 (control group). All animals in group 2 exhibit a slight decrease in CD4-positive cells and recover to baseline levels over time. A similartrend is observed in group 4 animals (Figure 14).

Virus load data are almost the inverse of CD4 data. Virus load declines below the level of detection in ¾ group 2 animals (and in the one control animal that maintains its CD4-positive cells), and the fourth animal shows only marginal virus load. Most of the other animals maintain a high or intermediate virus load (Figure 15).

Surprisingly, anti-Tat and anti-Nef antibody titres measured by ELISA were 2 to 3-fold higher in Group 3 (with mutated Tat) than in Group 5 (the equivalent Group with non-mutated Tat) throughout the course of the study.

At week 68 (56 weeks post challenge) all animals from the groups that had received the full antigen combination (groups 2 and 4) were still alive, while most of the animals in the other groupshad to be euthanized due to AIDS-like symptoms. The surviving animals per group were:

Group 1:	2/4
Group 2:	4/4
Group 3:	0/4
Group 4	4/4
Group 5	0/4
Group 6	1/4

Conclusions

The combination of gp120 and NefTat (in the presence of SIV Nef) prevents the loss of CD4-positive cells, reduces the virus load in animals infected with pathogenic $SHIV_{89.6p}$, and delays or prevents the development of AIDS-like disease symptoms, while gp120 or NefTat/SIV Nef alone do not protect from the pathologic consequences of the SHIV challenge.

The adjuvant 2 which is an oil in water emulsion comprising squalene, tocopherol and Tween 80, together with 3D-MPL and QS21 seems to have a stronger effect on the study endpoints than the alum / CpG adjuvant.

Second study

A second rhesus monkey SHIV challenge study was conducted to confirm the efficacy of the candidate vaccine gp120/NefTat + adjuvant and to compare different Tat-based antigens. The study was conducted by a different laboratory.

The design of the study was as follows.

Groups of 6 rhesus monkeys were immunized at 0, 4 and 12 weeks with injections i.m. and challenged at week 16 with a standard dose of pathogenic $SHIV_{89.6p}$.

Group 1 is the repeat of Group 2 in the first study.

Group 1:	Adjuvant 2	+ gp120	+ NefTat	+ SIV Nef
Group 2:	Adjuvant 2	+ gp120	+ Tat (oxidised)	
Group 3:	Adjuvant 2	+ gp120	+ Tat (reduced)	
Group 4	Adjuvant 2			

The follow-up/endpoints were again % CD4-positive cells, virus load by RT-PCR, morbidity and mortality

Results

All animals except one in group 2 become infected after challenge with $SHIV_{89.6p}$.

CD4-positive cells decline significantly after challenge in all animals of control group 4 and group 3, and in all but one animals of group 2. Only one animal in group 1 shows a marked decrease in CD4-positive cells. Unlike the animals from the first study, the monkeys in the second experiment display a stabilisation of CD4-positive cells at different levels one month after virus challenge (Figure 16). The stabilisation is generally lower than the initial % of CD4-positive cells, but will never lead to a complete loss of the cells. This may be indicative of a lower susceptibility to SHIV-induced disease in the monkey population that was used for the second study. Nonetheless, a beneficial effect of the gp120/NefTat/SIV Nef vaccine and the two gp120/Tat vaccines is demonstrable. The number of animals with a % of CD4-positive cells above 20 is 5 for the vaccinated animals, while none of the control animals from the adjuvant group remains above that level.

Analysis of RNA plasma virus loads confirms the relatively low susceptibility of the study animals (Figure 17). Only 2 of the 6 control animals maintain a high virus load, while the virus disappears from the plasma in the other animals. Thus, a vaccine effect is difficult to demonstrate for the virus load parameter.

Conclusions

Analysis of CD4-positive cells indicates that the vaccine gp120/NefTat + adjuvant (in the presence of SIV Nef) prevents the drop of CD4-positive cells in most vaccinated

animals This is a confirmation of the result obtained in the first SHIV study. Due to the lack of susceptibility of the study animals, the virus load parameter could not be used to demonstrate a vaccine effect. Taken together, the combination of gp120 and Tat and Nef HIV antigens provides protection against the pathologic consequences of HIV infection, as evidenced in a SHIV model.

The Tat alone antigens in combination with gp120 also provide some protection from the decline of CD4-positive cells. The effect is less pronounced than with the gp120/NefTat/SIV Nef antigen combination, but it demonstrates that gp120 and Tat are able to mediate some protective efficacy against SHIV-induced disease manifestations.

The second SHIV challenge study was performed with rhesus monkeys from a source completely unrelated to the source of animals from the first study. Both parameters, % of CD4-positive cells and plasma virus load, suggest that the animals in the second study were less susceptible to SHIV-induced disease, and that there was considerably greater variability among the animals. Nonetheless, a beneficial effect on the maintenance of CD4-positive cells of the gp120/NefTat/SIV Nef vaccine was seen with the experimental vaccine containing gp120/NefTat and SIV Nef. This indicates that the vaccine effect was not only repeated in a separate study, but furthermore demonstrated in an unrelated monkey population.

CLAIMS

1. Use of a) an HIV Tat protein or polynucleotide; or

b) an HIV Nef protein or polynucleotide; or

c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or polynucleotide (Nef-Tat);

and an HIV gp120 protein or polynucleotide in the manufacture of a vaccine for the prophylactic or therapeutic immunisation of humans against HIV.

2. Use as claimed in claim 1 wherein the Tat, Nef or Nef-Tat act in synergy with gp120 in the treatment or prevention of HIV.

3. Use as claimed in claim 1 or claim 2 wherein the vaccine in use reduces the HIV viral load in HIV infected humans.

4. Use as claimed in claims 1 or 2 wherein the vaccine in use results in a maintenance of CD4+ levels over those levels found in the absence of vaccination with HIV Tat, Nef or Nef-Tat and HIV gp120.

5. Use as claimed in any one of claims 1 – 4 wherein the vaccine further comprises an antigen selected from the group consisting of: gag, rev, vif, vpr, vpu.

6. Use as claimed in any one of claims 1 – 5 wherein the Tat protein is a mutated protein.

7. Use as claimed in any one of claims 1 – 6 wherein the Tat, Nef or Nef-Tat protein is reduced.

8. Use as claimed in any one of claims 1 – 7 wherein the Tat, Nef or Nef-Tat protein is carbamidomethylated.

9. Use as claimed in any one of claims 1 – 6 wherein the Tat, Nef or Nef-Tat protein is oxidised.

10. Use as claimed in any one of claims 1 – 9 which additionally comprises an adjuvant.

11. Use as claimed in claim 10 wherein the adjuvant is a TH1 inducing adjuvant.

12. Use as claimed in claim 10 or claim 11 wherein the adjuvant comprises monophosphoryl lipid A or a derivative thereof such as 3-de-O-acylated monophsphoryl lipid A.

13. Use as claimed in any one of claims 10 – 12 additionally comprising a saponin adjuvant.

14. Use as claimed in any one of claims 10 – 13 additionally comprising an oil in water emulsion.

15. Use as claimed in claim 10 or claim 11 wherein the adjuvant comprises CpG motif-containing oligonucleotides.

16. Use as claimed in claim 15 further comprising an aluminium salt.

17. Use of a) an HIV Tat protein or polynucleotide; or

b) an HIV Nef protein or polynucleotide; or

c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or polynucleotide;

and an HIV gp120 protein or polynucleotide in the manufacture of a vaccine suitable for a prime-boost delivery for the prophylactic or therapeutic immunisation of humans against HIV.

18. A method of immunising a human against HIV by administering to the human a vaccine comprising HIV Tat or HIV Nef or HIV NefTat in combination with HIV gp120 proteins or polynucleotides encoding them.

19. A vaccine composition for human use which vaccine composition comprises HIV Tat or HIV Nef or HIV Nef-Tat in combination with HIV gp120 proteins or polynucleotides encoding them.

FIGURE 1

The DNA and amino acid sequences of Nef-His; Tat-His; Nef-Tat-His fusion and mutated Tat is illustrated.

Pichia-expressed constructs (plain constructs)

⇒ *Nef - HIS*

DNA sequence (Seq. ID. No. 8)

ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGA
ATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAA
AAACATGGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGG
CTAGAAGCACAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTA
AGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGG
GGACTGGAAGGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATC
TACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTC
AGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAG
GTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCAT
GGAATGGATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCA
TTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGC
CACCATCACCATCACCATTAA

Protein sequence(Seq. ID. No. 9)

MGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAW
LEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWI
YHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLH
GMDDPEREVLEWRFDSRLAFHHVARELHPEYFKNCTSGHHHHH.

⇒ *Tat - HIS*

DNA sequence (Seq. ID. No. 10)

ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAA
ACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTC
ATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGA
CCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAA

TCCCGAGGGGACCCGACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATCACCAT
TAA

Protein sequence (Seq. ID. No. 11)

MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRR
PPQGSQTHQVSLSKQPTSQSRGDPTGPKETSGHHHHHH.

⇒ *Nef - Tat - HIS*

DNA sequence (Seq. ID. No. 12)

ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGA
ATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAA
AAACATGGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGG
CTAGAAGCACAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTA
AGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGG
GGACTGGAAGGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATC
TACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTC
AGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAG
GTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCAT
GGAATGGATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCA
TTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAG
CCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACTGCT
TGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACA
AAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCT
CAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCCGA
GGGGACCCGACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATCACCATTAA

Protein sequence(Seq. ID. No. 13)

MGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAW
LEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWI
YHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLH
GMDDPEREVLEWRFDSRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTA
CTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSR
GDPTGPKETSGHHHHHH.

E.coli-expressed constructs (fusion constructs)

⇒ *LipoD-Nef-HIS*

DNA sequence (Seq. ID. No. 14)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.
The Lipidation Signal Sequence is underlined. After processing, the cysteine coded by the TGT codon, indicated with a star, becomes the amino terminal residue which is then modified by covalently bound fatty acids.

ATGGATCCAAAAACTTTAGCCCTTTCTTTATTAGCAGCTGGCGTACTAGCAGGTTGT
AGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAAATCATTATT
GCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCTAAAGCACTT
GCTTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACTAAGGATGGT
CGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAA
TTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAA
GAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGCAAGTGGTCA
AAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCA
GCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGAGCAATCACA
AGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCACAAGAGGAG
GAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAG
GCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAATT
CACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC
TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTT
GGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAA
GGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGATGACCCTGAG
AGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCACGTGGCCCGA
GAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGCCACCATCACCATCACCAT
TAA

Protein sequence of the processed lipidated ProtD-Nef-HIS protein (Seq. ID. No. 15)

(Amino-acids corresponding to Prot D fusion partner are in bold)

CSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYLEQDLAMTKD
GRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMGGKW
SKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQE
EEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQG
YFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDP
EREVLEWRFDSRLAFHHVARELHPEYFKNCTSGHHHHHH.

⇒ *LipoD-Nef-Tat-HIS*

DNA sequence (Seq. ID. No. 16)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.
The Lipidation Signal Sequence is underlined. After processing, the cysteine coded by the TGT codon, indicated with a star, becomes the amino terminal residue which is then modified by covalently bound fatty acids.

ATGGATCCAAAAACTTTAGCCCTTTCTTTATTAGCAGCTGGCGTACTAGCAGGTTGT
AGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAAATCATTATT
GCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCTAAAGCACTT
GCGTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACTAAGGATGGT
CGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAA
TTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAA
GAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGCAAGTGGTCA
AAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCA
GCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGAGCAATCACA
AGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCACAAGAGGAG
GAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAG
GCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAATT
CACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC
TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTT
GGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAA
AGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCACGTGGCCCGA
GAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGATCCTAGACTA
GAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACTGCTTGTACCAATTGCTATTGT
AAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACAAAAGCCTTAGGCATCTCC
TATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCAT
CAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCCGAGGGGACCCGACAGGCCCG
AAGGAAACTAGTGGCCACCATCACCATCACCATTAA

Protein sequence of the processed lipidated ProtD-NEF-TAT-HIS protein (Seq. ID. No. 17)

(Amino-acids corresponding to Prot D fusion partner are in bold)

CSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYLEQDLAMTKD
GRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMGGKW
SKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQE
EEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQG
YFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDP
EREVLEWRFDSRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTACTNCY
PKETSGHHHHHH.

⇒ *ProtD-Nef -HIS*

DNA sequence (Seq. ID. No. 18)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAA
ATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCT
AAAGCACTTGCGTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACT
AAGGATGGTCGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTT
GCGAAAAAATTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTT
ACCTTAAAAGAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGC
AAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGA
GCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGA
GCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCA
CAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATG
ACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAA
GGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACA
CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCA
CTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAG
GCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGAT
GACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCAC
GTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGCCACCATCAC
CATCACCATTAA

Protein sequence (Seq. ID. No. 19)

(Amino-acids corresponding to Prot D fusion partner are in bold)

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYL
EQDLAMTKDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLK
EIQSLEMTENFETMGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDL
EKHGAITSSNTAATNAACAWLEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSH
FLKEKGGLEGLIHSQRRQDILDLWIYHTQGYFPDWQNYTPGPGVRYPLTFGW
CYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDPEREVLEWRFDSRLAFH
HVARELHPEYFKNCTSGHHHHHH.

⇒ *ProtD-Nef -Tat-HIS*

DNA sequence (Seq. ID. No. 20)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAA
ATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCT
AAAGCACTTGCGTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACT
AAGGATGGTCGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTT
GCGAAAAAATTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTT
ACCTTAAAAGAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGC
AAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGA
GCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGA
GCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCA
CAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATG
ACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAA
GGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACA
CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCA
CTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAG
GCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGAT
GACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCAC
GTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGAT
CCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACTGCTTGTACCAAT
TGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACAAAAGCCTTA
GGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGT
CAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCCGAGGGGACCCG
ACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATCACCATTAA

Protein sequence (Seq. ID. No. 21)

(Amino-acids corresponding to Prot D fusion partner are in bold)

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYLEQDLAMT
KDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMGG
KWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEA
QEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHT
QGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMD
DPEREVLEWRFDSRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTACTN
CYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDP
TGPKETSGHHHHHH.

⇒ Tat-MUTANT-HIS

DNA sequence (Seq. ID. No. 22)

```
ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATC   40
CAGGAAGTCAGCCTAAAACTGCTTGTACCAATTGCTATTG   80
TAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACA  120
GCTGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGAC  160
AGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGT  200
TTCTCTATCAAAGCAACCCACCTCCCAATCCAAAGGGGAG  240
CCGACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATC  280
ACCATTAA                                  288
```

Protein sequence(Seq. ID. No. 23)

Mutated amino-acids in Tat sequences are in bold.

```
MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFIT   40
AALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSKGE   80
PTGPKETSGHHHHHH.                           95
```

⇒Nef-Tat-Mutant-HIS

DNA sequence(Seq. ID. No. 24)

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ACCTCCCAATCCAAAGGGGAGCCGACAGGCCCGAAGGAAA  880
CTAGTGGCCACCATCACCATCACCATTAA              909
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Protein sequence (Seq. ID. No. 25)

Mutated amino-acids in Tat sequence are in bold.

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FPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGE 160
NTSLLHPVSLHGMDDPEREVLEWRFDSRLAFHHVARELHP 200
EYFKNCTSEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFH 240
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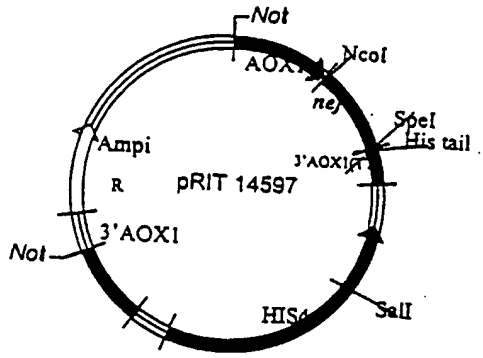
Figure 2

Map of pRIT14597 integrative vector



MCS POLYLINKER: *nef* gene inserted between NcoI and SpeI sites.

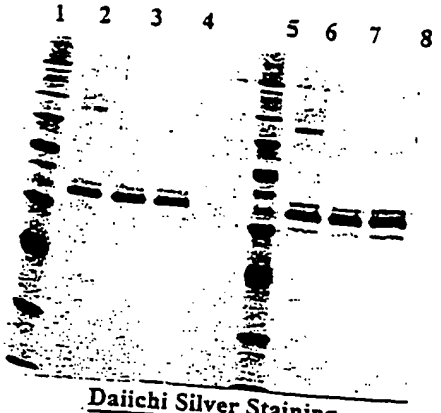
Asu II *Nco* I *Spe* I *Eco* RI

TTCGAA.ACC.ATGGCCGCGGACTAGTGGC.CAC.CAT.CAC.CAT.CAC.CAT.TAA.CGGAATTC

Thr .Ser . Gly. His . His . His . His . His . His

The amino acid sequence of Figure 2 relates to Seq. ID no. 27 and the nucleic acid sequence of Figure 3 relates to Seq. ID. No. 26.

Figure 3 : SDS-PAGE: Nef-Tat-his fusion protein

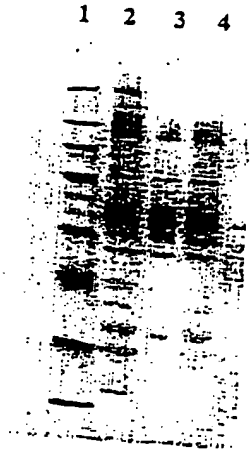


Daiichi Silver Staining

1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa
2: TNH/23 SP eluate (250 ng)
3: TNH/23 Purified bulk (250 ng)
4: TNH/22 Purified bulk (250 ng)

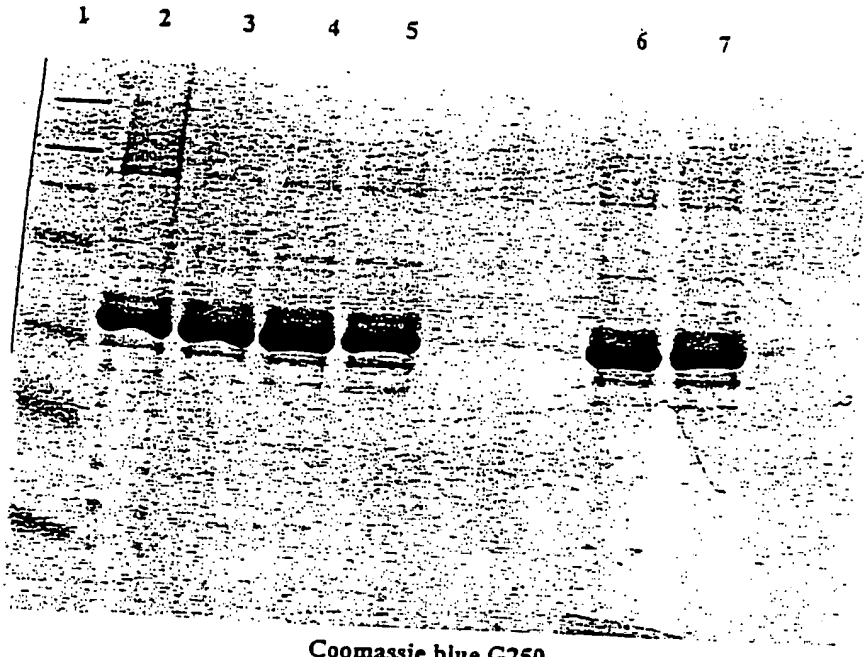
5: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa
6: TNH/23 SP eluate (400 ng)
7: TNH/23 Purified bulk (400 ng)
8: TNH/22 Purified bulk (400 ng)

Blot α Nef-Tat (LAS 97340)



Blot Tat2

Figure 4 : SDS-PAGE: Nef-Tat-his fusion protein

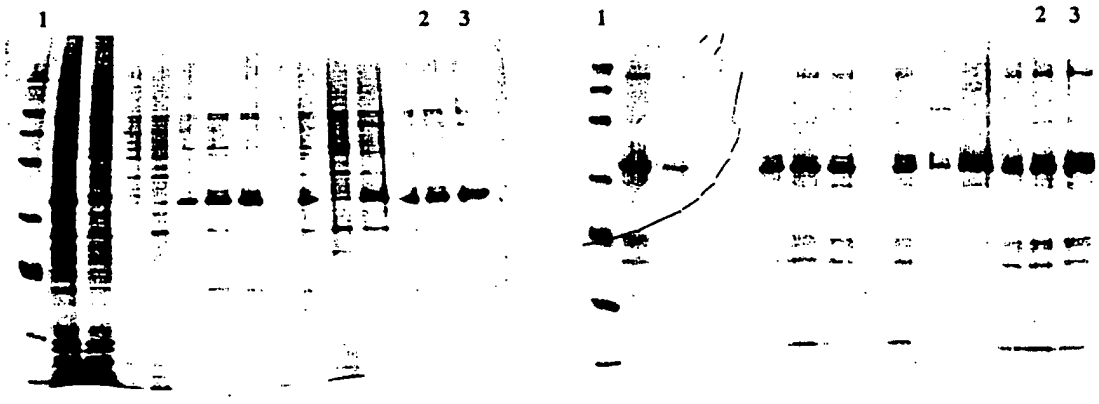


Coomassie blue G250

1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)
2: TNH/23 SP eluate (4 µg)
3: TNH/23 Superdex200 eluate (4 µg)
4: TNH/23 Purified bulk (4 µg)
5: TNH/22 Purified bulk (4 µg)

6: TNH/23 Purified bulk (4 µg) / non reducing conditions
7: TNH/22 Purified bulk (4 µg) / non reducing conditions

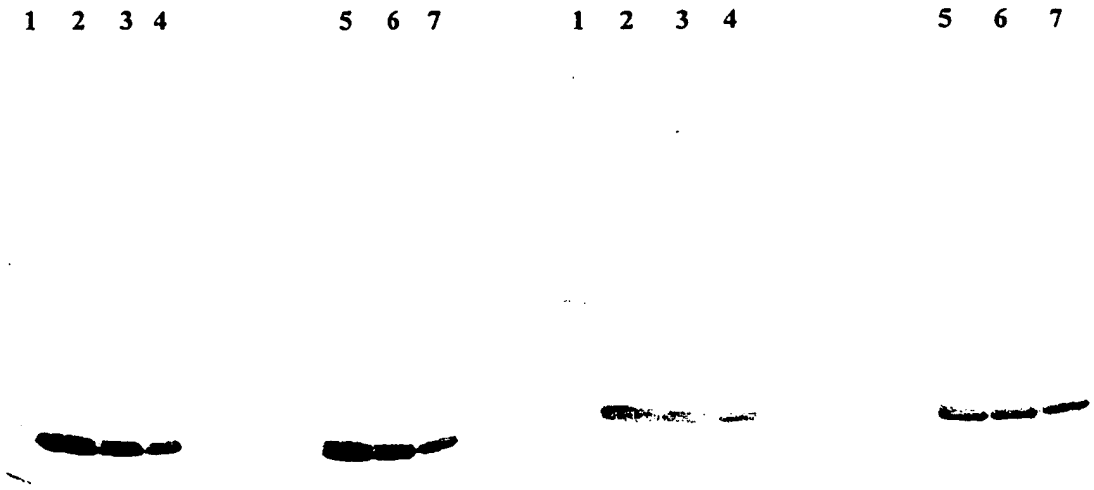
Figure 6: SDS-PAGE ANALYSIS – reducing conditions (14% polyacrylamide precasted gels - Novex) See example 5



Silver staining Western blot α Tat

1: MW (175/83/62/47,5/32,5/25/16,5/6,5 kDa)
2: Purified bulk
3: Purified bulk

Figure 7 (relating to Example 6): SDS-PAGE ANALYSIS:
(4-20% polyacrylamide precasted gels - Novex)

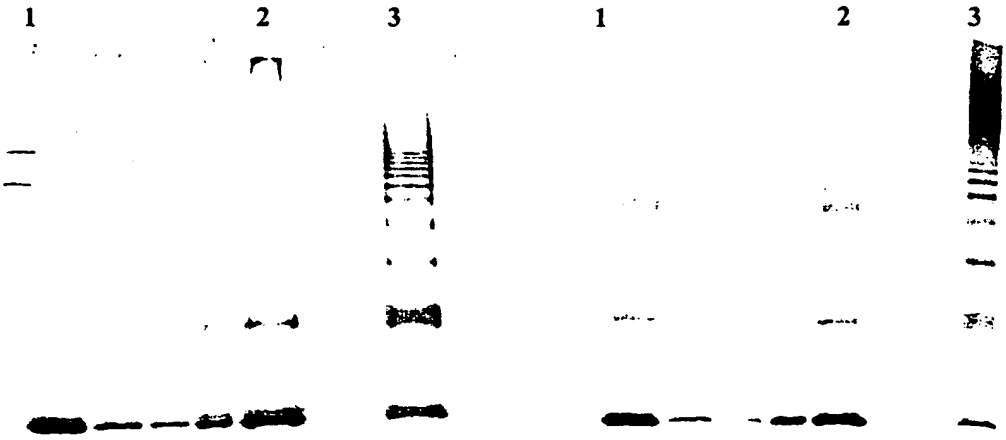


Coomassie blue G250 **Western blot anti Tat**

1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)
2: Purified bulk (reducing conditions)
3: Purified bulk (reducing conditions)
4: Purified bulk (reducing conditions)

5: Purified bulk (non reducing conditions)
6: Purified bulk (non reducing conditions)
7: Purified bulk (non reducing conditions)

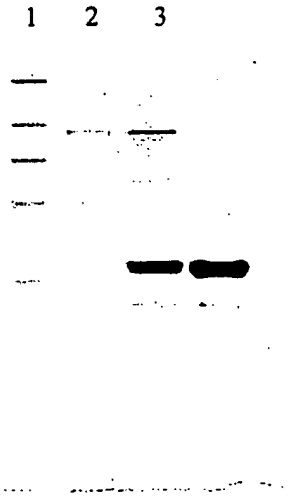
Figure 8 (relating to Example 7): SDS-PAGE ANALYSIS:
(4-20% pc lyacrylamide precasted gels - Novex)



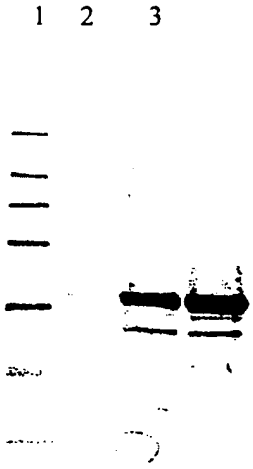
Coomassie blue G250 **Western blot anti Tat**

1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)
2: Purified bulk (reducing conditions)
3: Purified bulk (non reducing conditions)

FIGURE 9: SDS-PAGE ANALYSIS - REDUCING CONDITIONS
(14% polyacrylamide precasted gels - Novex) see Example 8

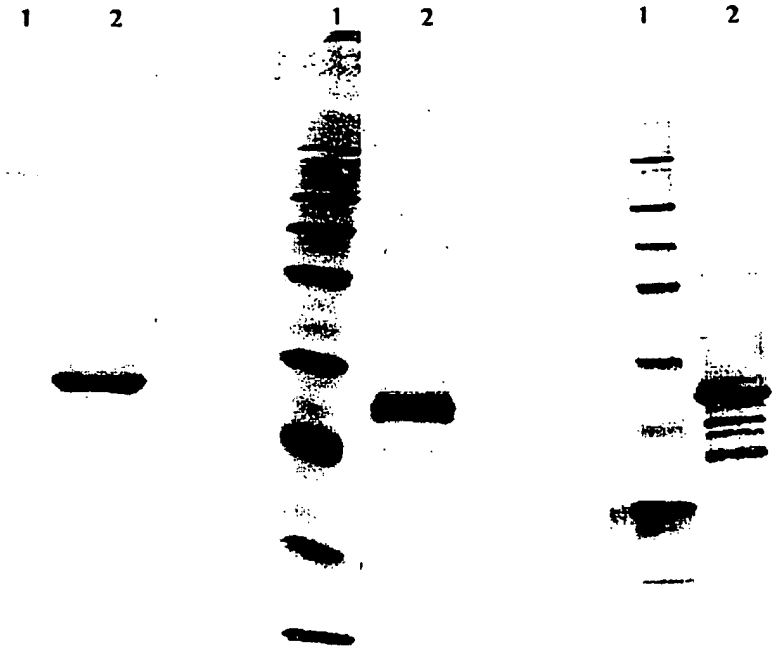


Coomassie blue R250



Silver staining

Figure 10: SDS-PAGE ANALYSIS – REDUCING CONDITIONS
(14% polyacrylamide precasted gels - Novex) See Example 9

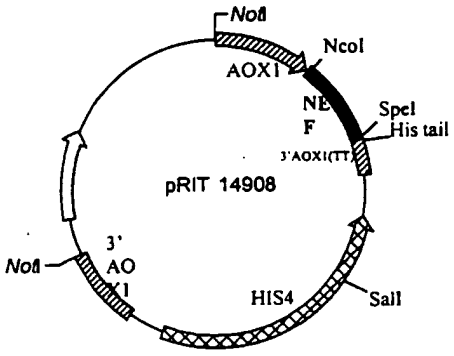


Coomassie blue G250 **Silver staining** **W. Blot α Nef**

1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)
2: Purified bulk

Figure 11

Map of pRIT14908 integrative vector



MCS POLYLINKER : NEF gene inserted between NcoI and SpeI sites.

Asu II *Nco* I *Spe* I *Eco* RI

TTCGAA.A CC.ATG GCCGCGG ACTAGT .GGC.CAC.CAT.CAC.CAT.CAC.CAT.TAA.CGC GAATTC

Thr .Ser . Gly. His . His . His . His . His . His .

Figure 12

Sequences of Pichia-expressed SIV-NEF-His protein

DNA SEQUENCE:

```
atgggtggagctatttccatgaggcggtccaggccgtctggagatctgcg    50
acagagactcttgcgggcgcgtggggagacttatgggagactcttaggag   100
aggtggaagatggatactcgcaatccccaggaggattagacaagggcttg   150
agctcactctcttgtgagggacagaaatacaatcagggacagtatatgaa   200
tactccatggagaaacccagctgaagagagagaaaaattagcatacagaa   250
aacaaaatatggatgatatagatgaggaagatgatgacttggtaggggta   300
tcagtgaggccaaaagttcccctaagaacaatgagttacaaattggcaat   350
agacatgtctcatttataaaagaaaaggggggactggaagggatttatt    400
acagtgcaagaagacatagaatcttagacatatacttagaaaaggaagaa   450
ggcatcataccagattggcaggattacacctcaggaccaggaattagata   500
cccaaagacatttggctggctatggaaattagtccctgtaaatgtatcag   550
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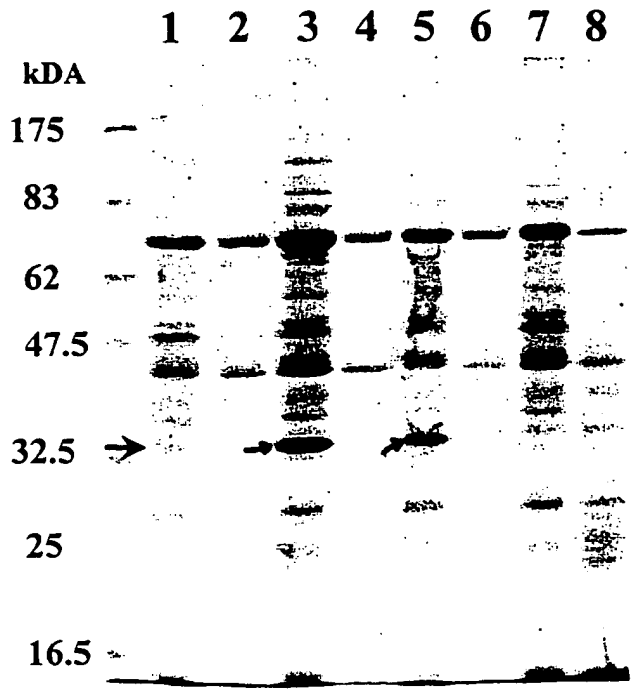
tcccagtgggatgacccttggggagaggttctagcatggaagtttgatcc   650
aactctggcctacacttatgaggcatatgttagatacccagaagagtttg   700
gaagcaagtcaggcctgtcagaggaagaggttagaagaaggctaaccgca   750
agaggccttcttaacatggctgacaagaaggaaactcgcactagtggcca   800
ccatcaccatcaccattaa.                                 819
```

PROTEIN SEQUENCE:

```
MGGAISMRRSRPSGDLRQRLLRARGETYGRLLGEVEDGYSQSPGGLDKGL    50
SSLSCEGQKYNQGQYMNTPWRNPAEEREKLAYRKQNMDDIDEEDDDLVGV   100
SVRPKVPLRTMSYKLAIDMSHFIKEKGGLEGIYYSARRHRILDIYLEKEE   150
GIIPDWQDYTSGPGIRYPKTFGWLWKLVPVNVSDEAQEDEEHYLMHPAQT   200
SQWDDPWGEVLAWKFDPTLAYTYEAYVRYPEEFGSKSGLSEEEVRRRLTA   250
RGLLNMADKKETRTSGHHHHHH.                               272
```

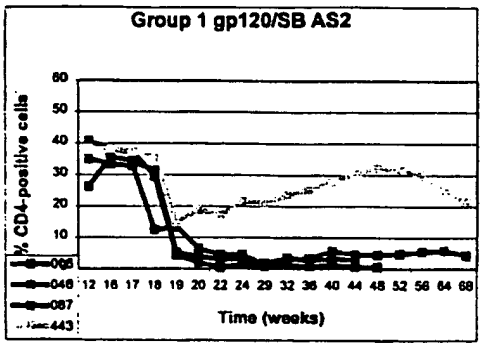
Figure 13

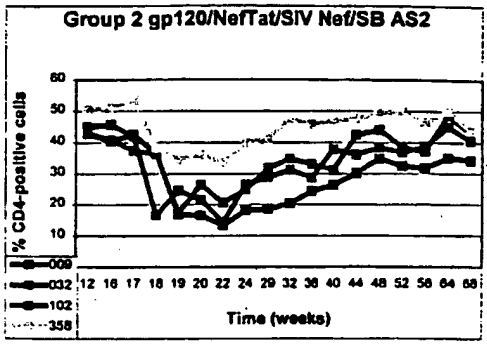
Coomassie Blue Stained SDS-PAGE of recombinant Pichia pastoris SIV/NEF expressing strains

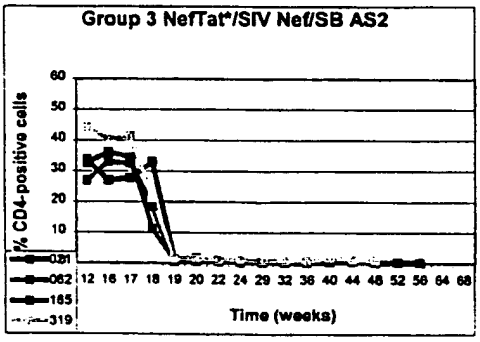


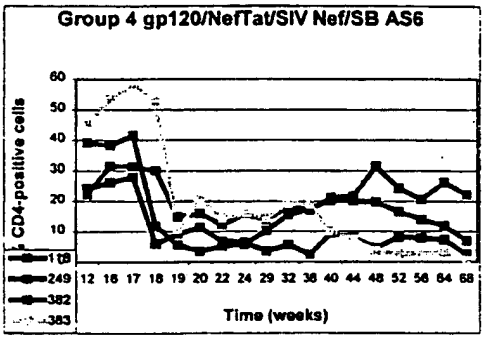
lane 1: P- Y1752 strain
lane 2: S- " "
lane 3: P- Y1772 strain
lane 4: S- " " "
lane 7: P- GS115 strain (negative control)
lane 8: S- " "

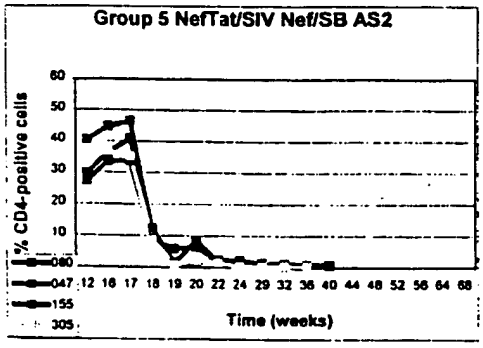
Figure 14. Monkey study 1. Analysi: of CD4-positive cells among PBMCs before and after challenge with SHIV











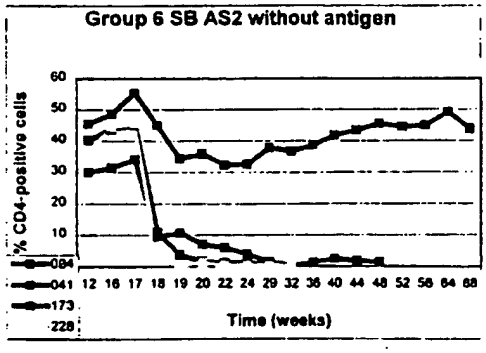
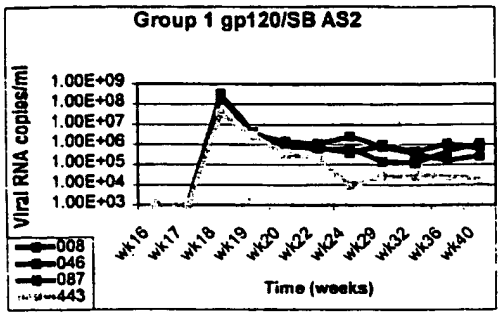
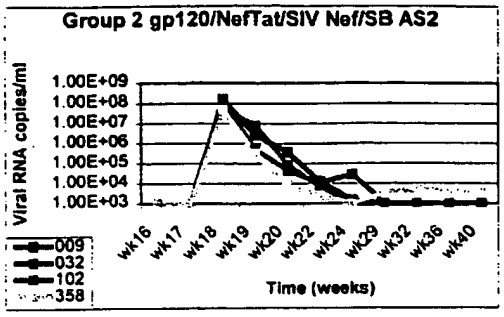
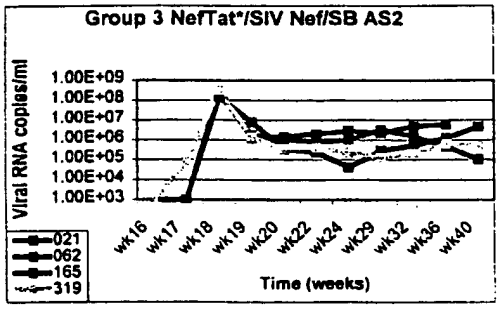
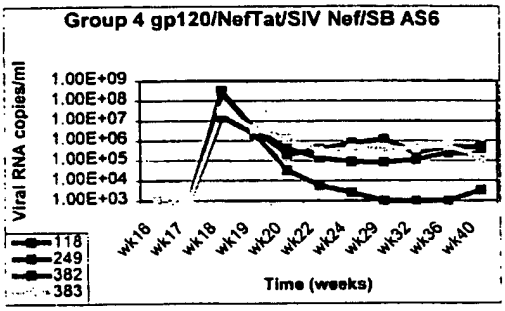


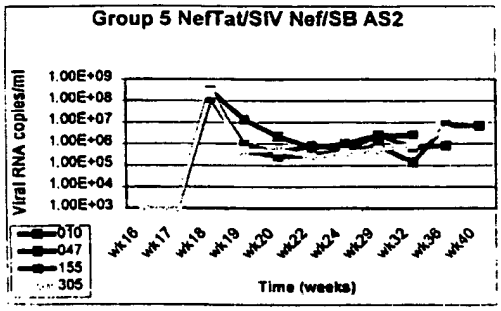
Figure 15. Monkey study 1. Analysis of SHIV plasma virus load after challenge with SHIV











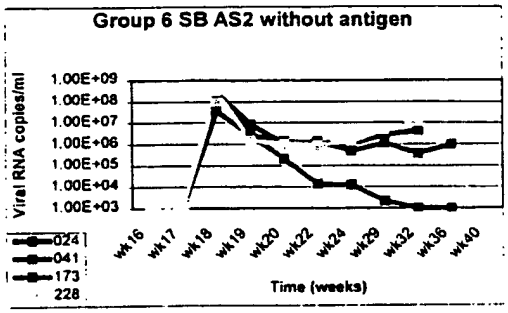


Figure 16. Monkey study 2. Analysis of CD4-positive cells among PBMCs before and after challenge with SHIV

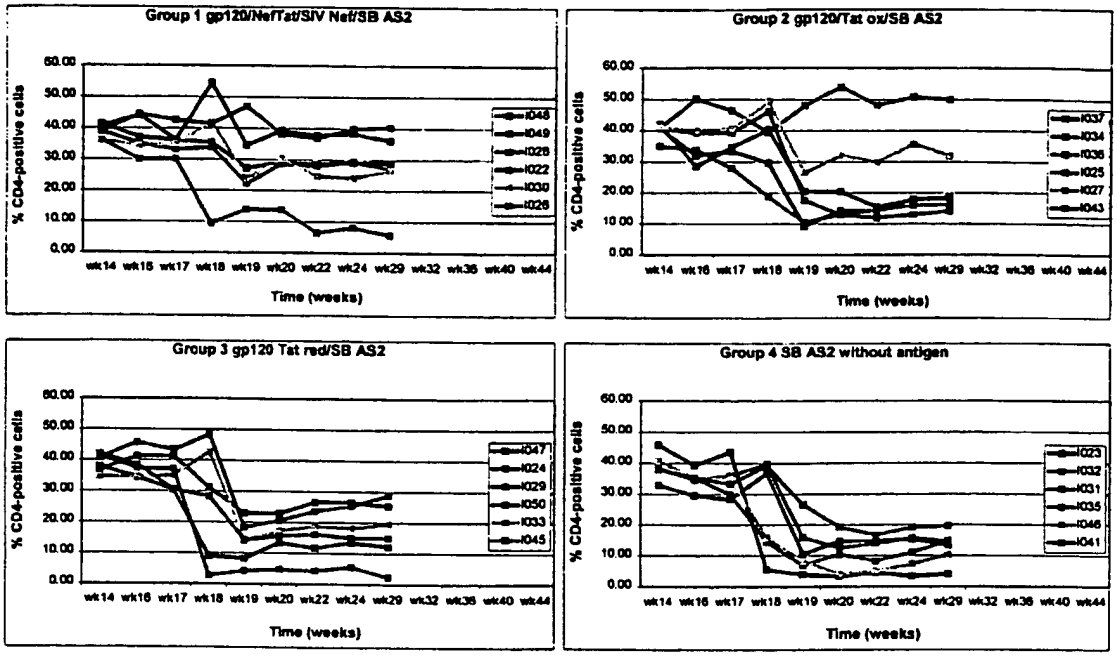
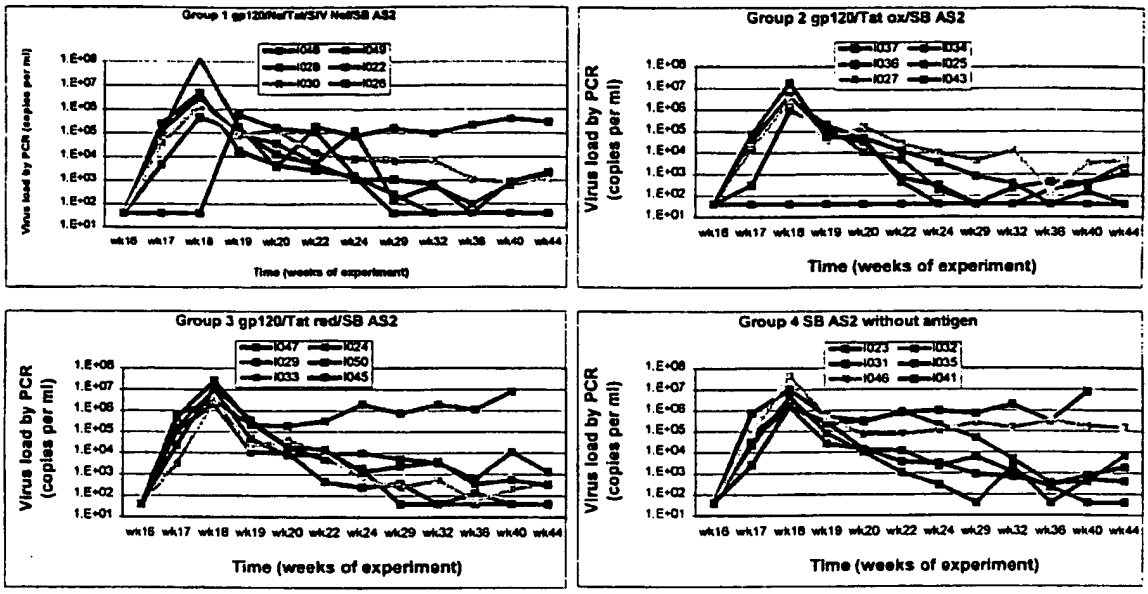


Figure 17. Monkey study 2. Analysis of SHIV plasma virus load after challenge with SHIV



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aacaccagct tgttacaccc tgtgagcctg catggaatgg atgaccctga gagagaagtg 540
ttagagtgga ggtttgacag ccgcctagca tttcatcacg tggcccgaga gctgcatccg 600
gagtacttca agaactgcac tagtggccac catcaccatc accattaa 648

<210> 9
<211> 215
<212> PRT
<213> human

<400> 9
Met Gly Gly Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val
1 5 10 15
Arg Glu Arg Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala
20 25 30
Ala Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr
35 40 45
Ala Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu
50 55 60
Glu Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr
65 70 75 80
Tyr Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly
85 90 95
Leu Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu

```
            100                 105                 110
Trp Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr
        115                 120                 125
Pro Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys
    130                 135                 140
Leu Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu
145                 150                 155                 160
Asn Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro
                165                 170                 175
Glu Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His
            180                 185                 190
His Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser
        195                 200                 205
Gly His His His His His His
    210                 215
```

<210> 10
<211> 288
<212> DNA
<213> human

<400> 10

```
atggagccag tagatcctag actagagccc tggaagcatc caggaagtca gcctaaaact   60
gcttgtacca attgctattg taaaaagtgt tgctttcatt gccaagtttg tttcataaca   120
aaagccttag gcatctccta tggcaggaag aagcggagac agcgacgaag acctcctcaa   180
ggcagtcaga ctcatcaagt ttctctatca aagcaaccca cctcccaatc ccgaggggac   240
ccgacaggcc cgaaggaaac tagtggccac catcaccatc accattaa                288
```

<210> 11
<211> 95
<212> PRT
<213> human

<400> 11

```
Met Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser
 1               5                  10                  15
Gln Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe
            20                  25                  30
His Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly
        35                  40                  45
Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr
    50                  55                  60
His Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp
65                  70                  75                  80
Pro Thr Gly Pro Lys Glu Thr Ser Gly His His His His His His
                85                  90                  95
```

<210> 12
<211> 909
<212> DNA
<213> human

<400> 12

```
atgggtggca agtggtcaaa aagtagtgtg gttggatggc ctactgtaag ggaaagaatg   60
agacgagctg agccagcagc agatggggtg ggagcagcat ctcgagacct ggaaaaacat   120
ggagcaatca caagtagcaa tacagcagct accaatgctg cttgtgcctg gctagaagca   180
caagaggagg aggaggtggg ttttccagtc acacctcagg tacctttaag accaatgact   240
tacaaggcag ctgtagatct tagccacttt ttaaaagaaa aggggggact ggaagggcta   300
attcactccc aacgaagaca agatatcctt gatctgtgga tctaccacac acaaggctac   360
ttccctgatt ggcagaacta cacaccaggg ccaggggtca gatatccact gacctttgga   420
tggtgctaca agctagtacc agttgagcca gataaggtag aagaggccaa taaaggagag   480
aacaccagct tgttacaccc tgtgagcctg catggaatgg atgaccctga gagagaagtg   540
ttagagtgga ggtttgacag ccgcctagca tttcatcacg tggcccgaga gctgcatccg   600
gagtacttca agaactgcac tagtgagcca gtagatccta gactagagcc ctggaagcat   660
```

```
ccaggaagtc agcctaaaac tgcttgtacc aattgctatt gtaaaaagtg ttgctttcat     720
tgccaagttt gtttcataac aaaagcctta ggcatctcct atggcaggaa gaagcggaga     780
cagcgacgaa gacctcctca aggcagtcag actcatcaag tttctctatc aaagcaaccc     840
acctcccaat cccgagggga cccgacaggc ccgaaggaaa ctagtggcca ccatcaccat     900
caccattaa                                                            909
```

<210> 13
<211> 302
<212> PRT
<213> human

<400> 13

```
Met Gly Gly Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val
 1               5                  10                  15
Arg Glu Arg Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala
            20                  25                  30
Ala Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr
        35                  40                  45
Ala Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu
    50                  55                  60
Glu Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr
65                  70                  75                  80
Tyr Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly
                85                  90                  95
Leu Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu
            100                 105                 110
Trp Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr
        115                 120                 125
Pro Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys
    130                 135                 140
Leu Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu
145                 150                 155                 160
Asn Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro
                165                 170                 175
Glu Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His
            180                 185                 190
His Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser
        195                 200                 205
Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln
    210                 215                 220
Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His
225                 230                 235                 240
Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly Arg
                245                 250                 255
Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr His
            260                 265                 270
Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp Pro
        275                 280                 285
Thr Gly Pro Lys Glu Thr Ser Gly His His His His His His
    290                 295                 300
```

<210> 14
<211> 1029
<212> DNA
<213> human

<400> 14

```
atggatccaa aaactttagc cctttcttta ttagcagctg gcgtactagc aggttgtagc      60
agccattcat caaatatggc gaatacccaa atgaaatcag acaaaatcat tattgctcac     120
cgtggtgcta gcggttattt accagagcat acgttagaat ctaaagcact tgcttttgca     180
caacaggctg attatttaga gcaagattta gcaatgacta aggatggtcg tttagtggtt     240
attcacgatc acttttaga tggcttgact gatgttgcga aaaaattccc acatcgtcat      300
cgtaaagatg gccgttacta tgtcatcgac tttaccttaa aagaaattca aagtttagaa     360
atgacagaaa actttgaaac catgggtggc aagtggtcaa aaagtagtgt ggttggatgg     420
```

```
cctactgtaa gggaaagaat gagacgagct gagccagcag cagatggggt gggagcagca      480
tctcgagacc tggaaaaaca tggagcaatc acaagtagca atacagcagc taccaatgct      540
gcttgtgcct ggctagaagc acaagaggag gaggaggtgg gttttccagt cacacctcag      600
gtacctttaa gaccaatgac ttacaaggca gctgtagatc ttagccactt tttaaaagaa      660
aaggggggac tggaagggct aattcactcc caacgaagac aagatatcct tgatctgtgg      720
atctaccaca cacaaggcta cttccctgat tggcagaact acacaccagg gccaggggtc      780
agatatccac tgacctttgg atggtgctac aagctagtac cagttgagcc agataaggta      840
gaagaggcca ataaaggaga gaacaccagc ttgttacacc ctgtgagcct gcatggaatg      900
gatgaccctg agagagaagt gttagagtgg aggtttgaca gccgcctagc atttcatcac      960
gtggcccgag agctgcatcc ggagtacttc aagaactgca ctagtggcca ccatcaccat     1020
caccattaa                                                             1029
```

<210> 15
<211> 324
<212> PRT
<213> human

<400> 15

```
Cys Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys Ser Asp
 1               5                  10                  15
Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro Glu His
            20                  25                  30
Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp Tyr Leu
        35                  40                  45
Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val Ile His
    50                  55                  60
Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe Pro His
65                  70                  75                  80
Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr Leu Lys
                85                  90                  95
Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met Gly Gly
            100                 105                 110
Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val Arg Glu Arg
        115                 120                 125
Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala Ala Ser Arg
    130                 135                 140
Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr Ala Ala Thr
145                 150                 155                 160
Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu Glu Val Gly
                165                 170                 175
Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr Tyr Lys Ala
            180                 185                 190
Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly Leu Glu Gly
        195                 200                 205
Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu Trp Ile Tyr
    210                 215                 220
His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr Pro Gly Pro
225                 230                 235                 240
Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys Leu Val Pro
                245                 250                 255
Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu Asn Thr Ser
            260                 265                 270
Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro Glu Arg Glu
        275                 280                 285
Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His His Val Ala
    290                 295                 300
Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Gly His His
305                 310                 315                 320
His His His His
```

<210> 16
<211> 1290
<212> DNA

<213> human

<400> 16

```
atggatccaa aaactttagc cctttcttta ttagcagctg gcgtactagc aggttgtagc      60
agccattcat caaatatggc gaatacccaa atgaaatcag acaaaatcat tattgctcac     120
cgtggtgcta gcggttattt accagagcat acgttagaat ctaaagcact tgcgtttgca     180
caacaggctg attatttaga gcaagattta gcaatgacta aggatggtcg tttagtggtt     240
attcacgatc acttttaga tggcttgact gatgttgcga aaaaattccc acatcgtcat      300
cgtaaagatg gccgttacta tgtcatcgac tttaccttaa aagaaattca aagtttagaa     360
atgacagaaa actttgaaac catgggtggc aagtggtcaa aaagtagtgt ggttggatgg     420
cctactgtaa gggaaagaat gagacgagct gagccagcag cagatggggt gggagcagca     480
tctcgagacc tggaaaaaca tggagcaatc acaagtagca atacagcagc taccaatgct     540
gcttgtgcct ggctagaagc acaagaggag gaggaggtgg gttttccagt cacacctcag     600
gtacctttaa gaccaatgac ttacaaggca gctgtagatc ttagccactt tttaaaagaa     660
aaggggggac tggaagggct aattcactcc caacgaagac aagatatcct tgatctgtgg     720
atctaccaca cacaaggcta cttccctgat tggcagaact acacaccagg gccaggggtc     780
agatatccac tgacctttgg atggtgctac aagctagtac cagttgagcc agataaggta     840
gaagaggcca ataaaggaga gaacaccagc ttgttacacc ctgtgagcct gcatggaatg     900
gatgaccctg agagagaagt gttagagtgg aggtttgaca gccgcctagc atttcatcac     960
gtggcccgag agctgcatcc ggagtacttc aagaactgca ctagtgagcc agtagatcct    1020
agactagagc cctggaagca tccaggaagt cagcctaaaa ctgcttgtac caattgctat    1080
tgtaaaaagt gttgctttca ttgccaagtt tgtttcataa caaaagcctt aggcatctcc    1140
tatggcagga agaagcggag acagcgacga agacctcctc aaggcagtca gactcatcaa    1200
gtttctctat caaagcaacc cacctcccaa tcccgagggg acccgacagg cccgaaggaa    1260
actagtggcc accatcacca tcaccattaa                                     1290
```

<210> 17
<211> 411
<212> PRT
<213> human

<400> 17

```
Cys Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys Ser Asp
 1               5                  10                  15
Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro Glu His
            20                  25                  30
Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp Tyr Leu
        35                  40                  45
Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val Ile His
    50                  55                  60
Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe Pro His
65                  70                  75                  80
Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr Leu Lys
                85                  90                  95
Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met Gly Gly
            100                 105                 110
Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val Arg Glu Arg
        115                 120                 125
Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala Ala Ser Arg
    130                 135                 140
Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr Ala Ala Thr
145                 150                 155                 160
Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu Glu Val Gly
                165                 170                 175
Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr Tyr Lys Ala
            180                 185                 190
Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly Leu Glu Gly
        195                 200                 205
Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu Trp Ile Tyr
    210                 215                 220
His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr Pro Gly Pro
225                 230                 235                 240
Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys Leu Val Pro
                245                 250                 255
```

```
Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu Asn Thr Ser
            260                 265                 270
Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro Glu Arg Glu
        275                 280                 285
Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His His Val Ala
    290                 295                 300
Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Glu Pro Val
305                 310                 315                 320
Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln Pro Lys Thr
                325                 330                 335
Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His Cys Gln Val
            340                 345                 350
Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly Arg Lys Lys Arg
        355                 360                 365
Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr His Gln Val Ser
    370                 375                 380
Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp Pro Thr Gly Pro
385                 390                 395                 400
Lys Glu Thr Ser Gly His His His His His His
                405                 410
```

<210> 18
<211> 981
<212> DNA
<213> human

<400> 18

```
atggatccaa gcagccattc atcaaatatg gcgaataccc aaatgaaatc agacaaaatc      60
attattgctc accgtggtgc tagcggttat ttaccagagc atacgttaga atctaaagca     120
cttgcgtttg cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatggt     180
cgtttagtgg ttattcacga tcacttttta gatggcttga ctgatgttgc gaaaaaattc     240
ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaatt     300
caaagtttag aaatgacaga aaactttgaa accatgggtg gcaagtggtc aaaaagtagt     360
gtggttggat ggcctactgt aagggaaaga atgagacgag ctgagccagc agcagatggg     420
gtgggagcag catctcgaga cctggaaaaa catggagcaa tcacaagtag caatacagca     480
gctaccaatg ctgcttgtgc ctggctagaa gcacaagagg aggaggaggt gggttttcca     540
gtcacacctc aggtaccttt aagaccaatg acttacaagg cagctgtaga tcttagccac     600
tttttaaaag aaaaggggag actggaaggg ctaattcact cccaacgaag acaagatatc     660
cttgatctgt ggatctacca cacacaaggc tacttccctg attggcagaa ctacacacca     720
gggccagggg tcagatatcc actgaccttt ggatggtgct acaagctagt accagttgag     780
ccagataagg tagaagaggc caataaagga gagaacacca gcttgttaca ccctgtgagc     840
ctgcatggaa tggatgaccc tgagagagaa gtgttagagt ggaggtttga cagccgccta     900
gcatttcatc acgtggcccg agagctgcat ccggagtact tcaagaactg cactagtggc     960
caccatcacc atcaccatta a                                               981
```

<210> 19
<211> 326
<212> PRT
<213> human

<400> 19

```
Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
 1               5                  10                  15
Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
            20                  25                  30
Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
        35                  40                  45
Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
    50                  55                  60
Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65                  70                  75                  80
Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
                85                  90                  95
Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
```

```
            100                 105                 110
Gly Gly Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val Arg
        115                 120                 125
Glu Arg Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala Ala
    130                 135                 140
Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr Ala
145                 150                 155                 160
Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu Glu
                165                 170                 175
Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr Tyr
            180                 185                 190
Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly Leu
        195                 200                 205
Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu Trp
    210                 215                 220
Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr Pro
225                 230                 235                 240
Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys Leu
                245                 250                 255
Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu Asn
            260                 265                 270
Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro Glu
        275                 280                 285
Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His His
    290                 295                 300
Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Gly
305                 310                 315                 320
His His His His His His
                325
```

<210> 20
<211> 1242
<212> DNA
<213> human

<400> 20

```
atggatccaa gcagccattc atcaaatatg gcgaataccc aaatgaaatc agacaaaatc      60
attattgctc accgtggtgc tagcggttat ttaccagagc atacgttaga atctaaagca     120
cttgcgtttg cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatggt     180
cgtttagtgg ttattcacga tcacttttta gatggcttga ctgatgttgc gaaaaaattc     240
ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaatt     300
caaagtttag aaatgacaga aaactttgaa accatgggtg gcaagtggtc aaaaagtagt     360
gtggttggat ggcctactgt aagggaaaga atgagacgag ctgagccagc agcagatggg     420
gtgggagcag catctcgaga cctggaaaaa catggagcaa tcacaagtag caatacagca     480
gctaccaatg ctgcttgtgc ctggctagaa gcacaagagg aggaggaggt gggttttcca     540
gtcacacctc aggtaccttt aagaccaatg acttacaagg cagctgtaga tcttagccac     600
tttttaaaag aaaaggggga actggaaggg ctaattcact cccaacgaag acaagatatc     660
cttgatctgt ggatctacca cacacaaggc tacttccctg attggcagaa ctacacacca     720
gggccagggg tcagatatcc actgaccttt ggatggtgct acaagctagt accagttgag     780
ccagataagg tagaaggagc caataaagga gagaacacca gcttgttaca ccctgtgagc     840
ctgcatggaa tggatgaccc tgagagagaa gtgttagagt ggaggtttga cagccgccta     900
gcatttcatc acgtggcccg agagctgcat ccggagtact tcaagaactg cactagtgag     960
ccagtagatc ctagactaga gccctggaag catccaggaa gtcagcctaa aactgcttgt    1020
accaattgct attgtaaaaa gtgttgcttt cattgccaag tttgtttcat aacaaaagcc    1080
ttaggcatct cctatggcag gaagaagcgg agacagcgac gaagacctcc tcaaggcagt    1140
cagactcatc aagtttctct atcaaagcaa cccacctccc aatcccgagg ggacccgaca    1200
ggcccgaagg aaactagtgg ccaccatcac catcaccatt aa                       1242
```

<210> 21
<211> 413
<212> PRT
<213> human

<400> 21

```
Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
 1               5                  10                  15
Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
            20                  25                  30
Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
        35                  40                  45
Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
    50                  55                  60
Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65                  70                  75                  80
Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
                85                  90                  95
Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
            100                 105                 110
Gly Gly Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val Arg
        115                 120                 125
Glu Arg Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala Ala
    130                 135                 140
Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr Ala
145                 150                 155                 160
Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu Glu
                165                 170                 175
Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr Tyr
            180                 185                 190
Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly Leu
        195                 200                 205
Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu Trp
    210                 215                 220
Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr Pro
225                 230                 235                 240
Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys Leu
                245                 250                 255
Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu Asn
            260                 265                 270
Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro Glu
        275                 280                 285
Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His His
    290                 295                 300
Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Glu
305                 310                 315                 320
Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln Pro
                325                 330                 335
Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His Cys
            340                 345                 350
Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly Arg Lys
        355                 360                 365
Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr His Gln
    370                 375                 380
Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp Pro Thr
385                 390                 395                 400
Gly Pro Lys Glu Thr Ser Gly His His His His His His
                405                 410
```

<210> 22
<211> 288
<212> DNA
<213> human

<400> 22

```
atggagccag tagatcctag actagagccc tggaagcatc caggaagtca gcctaaaact     60
gcttgtacca attgctattg taaaaagtgt tgctttcatt gccaagtttg tttcataaca    120
gctgccttag gcatctccta tggcaggaag aagcggagac agcgacgaag acctcctcaa    180
ggcagtcaga ctcatcaagt ttctctatca aagcaaccca cctcccaatc caaaggggag    240
ccgacaggcc cgaaggaaac tagtggccac catcaccatc accattaa                 288
```

<210> 23
<211> 95
<212> PRT
<213> human

<400> 23

```
Met Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser
 1               5                  10                  15
Gln Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe
            20                  25                  30
His Cys Gln Val Cys Phe Ile Thr Ala Ala Leu Gly Ile Ser Tyr Gly
        35                  40                  45
Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr
    50                  55                  60
His Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Lys Gly Glu
65                  70                  75                  80
Pro Thr Gly Pro Lys Glu Thr Ser Gly His His His His His His
                85                  90                  95
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<210> 24
<211> 909
<212> DNA
<213> human

<400> 24

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agacgagctg agccagcagc agatggggtg ggagcagcat ctcgagacct ggaaaaacat     120
ggagcaatca caagtagcaa tacagcagct accaatgctg cttgtgcctg gctagaagca     180
caagaggagg aggaggtggg ttttccagtc acacctcagg tacctttaag accaatgact     240
tacaaggcag ctgtagatct tagccacttt ttaaaagaaa aggggggact ggaagggcta     300
attcactccc aacgaagaca agatatcctt gatctgtgga tctaccacac acaaggctac     360
ttccctgatt ggcagaacta cacaccaggg ccaggggtca gatatccact gacctttgga     420
tggtgctaca agctagtacc agttgagcca gataaggtag aagaggccaa taaaggagag     480
aacaccagct tgttacaccc tgtgagcctg catggaatgg atgaccctga gagagaagtg     540
ttagagtgga ggtttgacag ccgcctagca tttcatcacg tggcccgaga gctgcatccg     600
gagtacttca agaactgcac tagtgagcca gtagatccta gactagagcc ctggaagcat     660
ccaggaagtc agcctaaaac tgcttgtacc aattgctatt gtaaaaagtg ttgctttcat     720
tgccaagttt gtttcataac agctgcctta ggcatctcct atggcaggaa gaagcggaga     780
cagcgacgaa gacctcctca aggcagtcag actcatcaag tttctctatc aaagcaaccc     840
acctcccaat ccaaagggga gccgacaggc ccgaaggaaa ctagtggcca ccatcaccat     900
caccattaa                                                            909
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<210> 25
<211> 302
<212> PRT
<213> human

<400> 25

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Met Gly Gly Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val
 1               5                  10                  15
Arg Glu Arg Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala
            20                  25                  30
Ala Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr
        35                  40                  45
Ala Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu
    50                  55                  60
Glu Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr
65                  70                  75                  80
Tyr Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly
                85                  90                  95
Leu Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu
            100                 105                 110
Trp Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr
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        115                 120                 125
Pro Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys
    130                 135                 140
Leu Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu
145                 150                 155                 160
Asn Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro
                165                 170                 175
Glu Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His
            180                 185                 190
His Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser
        195                 200                 205
Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln
    210                 215                 220
Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His
225                 230                 235                 240
Cys Gln Val Cys Phe Ile Thr Ala Ala Leu Gly Ile Ser Tyr Gly Arg
                245                 250                 255
Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr His
            260                 265                 270
Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Lys Gly Glu Pro
        275                 280                 285
Thr Gly Pro Lys Glu Thr Ser Gly His His His His His His
    290                 295                 300
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<210> 26
<211> 57
<212> DNA
<213> human

<400> 26
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<210> 27
<211> 9
<212> PRT
<213> human

<400> 27
Thr Ser Gly His His His His His His
 1   5

<210> 28
<211> 58
<212> DNA
<213> human

<400> 28
ttcgaaacca tggccgcgga ctagtggcca ccatcaccat caccattaac gcgaattc 58

<210> 29
<211> 9
<212> PRT
<213> human

<400> 29
Thr Ser Gly His His His His His His
 1   5

<210> 30
<211> 819
<212> DNA
<213> human

<400> 30

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ttgcgggcgc gtggggagac ttatgggaga ctcttaggag aggtggaaga tggatactcg     120
caatccccag gaggattaga caagggcttg agctcactct cttgtgaggg acagaaatac     180
aatcagggac agtatatgaa tactccatgg agaaacccag ctgaagagag agaaaaatta     240
gcatacagaa aacaaaatat ggatgatata gatgaggaag atgatgactt ggtaggggta     300
tcagtgaggc caaaagttcc cctaagaaca atgagttaca aattggcaat agacatgtct     360
cattttataa aagaaaaggg gggactggaa gggatttatt acagtgcaag aagacataga     420
atcttagaca tatacttaga aaaggaagaa ggcatcatac cagattggca ggattacacc     480
tcaggaccag gaattagata cccaaagaca tttggctggc tatggaaatt agtccctgta     540
aatgtatcag atgaggcaca ggaggatgag gagcattatt taatgcatcc agctcaaact     600
tcccagtggg atgacccttg gggagaggtt ctagcatgga agtttgatcc aactctggcc     660
tacacttatg aggcatatgt tagataccca gaagagtttg gaagcaagtc aggcctgtca     720
gaggaagagg ttagaagaag gctaaccgca agaggccttc ttaacatggc tgacaagaag     780
gaaactcgca ctagtggcca ccatcaccat caccattaa                            819
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<210> 31
<211> 272
<212> PRT
<213> human

<400> 31

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Met Gly Gly Ala Ile Ser Met Arg Arg Ser Arg Pro Ser Gly Asp Leu
 1               5                  10                  15
Arg Gln Arg Leu Leu Arg Ala Arg Gly Glu Thr Tyr Gly Arg Leu Leu
            20                  25                  30
Gly Glu Val Glu Asp Gly Tyr Ser Gln Ser Pro Gly Gly Leu Asp Lys
        35                  40                  45
Gly Leu Ser Ser Leu Ser Cys Glu Gly Gln Lys Tyr Asn Gln Gly Gln
    50                  55                  60
Tyr Met Asn Thr Pro Trp Arg Asn Pro Ala Glu Glu Arg Glu Lys Leu
65                  70                  75                  80
Ala Tyr Arg Lys Gln Asn Met Asp Asp Ile Asp Glu Glu Asp Asp Asp
                85                  90                  95
Leu Val Gly Val Ser Val Arg Pro Lys Val Pro Leu Arg Thr Met Ser
            100                 105                 110
Tyr Lys Leu Ala Ile Asp Met Ser His Phe Ile Lys Glu Lys Gly Gly
        115                 120                 125
Leu Glu Gly Ile Tyr Tyr Ser Ala Arg Arg His Arg Ile Leu Asp Ile
    130                 135                 140
Tyr Leu Glu Lys Glu Glu Gly Ile Ile Pro Asp Trp Gln Asp Tyr Thr
145                 150                 155                 160
Ser Gly Pro Gly Ile Arg Tyr Pro Lys Thr Phe Gly Trp Leu Trp Lys
                165                 170                 175
Leu Val Pro Val Asn Val Ser Asp Glu Ala Gln Glu Asp Glu Glu His
            180                 185                 190
Tyr Leu Met His Pro Ala Gln Thr Ser Gln Trp Asp Asp Pro Trp Gly
        195                 200                 205
Glu Val Leu Ala Trp Lys Phe Asp Pro Thr Leu Ala Tyr Thr Tyr Glu
    210                 215                 220
Ala Tyr Val Arg Tyr Pro Glu Glu Phe Gly Ser Lys Ser Gly Leu Ser
225                 230                 235                 240
Glu Glu Glu Val Arg Arg Arg Leu Thr Ala Arg Gly Leu Leu Asn Met
                245                 250                 255
Ala Asp Lys Lys Glu Thr Arg Thr Ser Gly His His His His His His
            260                 265                 270
```